

**SUBSTANCE P AND ITS ROLE  
IN SENSORY REFLEXES  
IN THE EYE**

Gunnel Bynke

Lund 1984



SUBSTANCE P AND ITS ROLE IN SENSORY REFLEXES IN THE EYE

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**Abstract** Intraocular inflammation is characterized by pupillary constriction and by breakdown of the blood-aqueous barrier with consequent protein leakage into the anterior chamber.  
The present study concerns the role of neurogenic mechanisms and of the neuropeptide substance P (SP) in particular in the response to ocular injury.  
The experiments were performed on the eyes of rabbits and on the isolated iris from rabbits. The breakdown of the blood-aqueous barrier was followed in vivo by measuring the leakage of protein into the aqueous humour by a photoelectric instrument. The pupillary constriction was studied in vivo and in vitro.  
The following results were obtained: SP in itself elicited protein leakage and pupillary constriction. These effects could not be prevented by the neuronal blocker tetrodotoxin, indicating that SP acts postjunctionally. Pretreatment with capsaicin, an SP depleting agent, prevented the protein leakage induced by subsequently applied local traumata. Pretreatment with tetrodotoxin prevented the response to infrared irradiation (IR) of the iris and to chemical irritants such as bradykinin, capsaicin and prostaglandins, indicating that the responses to these stimuli involve neurogenic mechanisms. Pretreatment with specific antagonists to SP inhibited the protein leakage and the pupillary constriction induced by IR of the iris or other irritant stimuli, e.g. SP, bradykinin, capsaicin and PGE<sub>2</sub>. These findings suggest that SP or a related peptide is involved in the ocular responses to irritant stimuli. Long-term treatment with SP antagonists reversibly abolished the protein leakage induced by IR, but did not affect corneal sensitivity. Hypersensitivity to SP occurred after long-term treatment with SP antagonists or tetrodotoxin. In sympathectomized rabbits, IR induced enhanced protein leakage, which could be abolished by SP antagonists. It is concluded that SP, or a related peptide, is a neurotransmitter in the anterior uvea, with an important role in the neurogenically mediated response to ocular trauma.

**Key words**  
Key words: intraocular inflammation-breakdown of blood-aqueous barrier-pupillary constriction-capsaicin-substance-P-substance P antagonists.

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From the Departments of Ophthalmology and Pharmacology,  
University of Lund, Lund, Sweden

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This thesis is based on the following papers, which will be referred to in the text by the Roman numerals assigned to them below.

- I Bynke G. 1983. Capsaicin pretreatment prevents disruption of the blood-aqueous barrier in the rabbit eye. Invest Ophthalmol. Vis Sci 24: 744-748.
- II Holmdahl\* G, Håkanson R, Leander S, Rosell S, Folkers K. & Sundler F. 1981. A substance P antagonist, (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP, inhibits inflammatory responses in the rabbit eye. Science 214: 1029-1031.
- III Bynke G, Håkanson R, Hörig J. & Leander S. 1983. Bradykinin contracts the pupillary sphincter and evokes ocular inflammation through release of neuronal substance P. Europ J Pharmacol 91: 469-475.
- IV Bynke G, Wahlestedt C & Håkanson R. Pupillary constriction by bradykinin and capsaicin: mode of action. Submitted to Europ J Pharmacol.
- V Bynke G, Håkanson R & Hörig J. 1983. Ocular responses evoked by capsaicin and prostaglandin E<sub>2</sub> are inhibited by a substance P antagonist. Experientia 39: 996-998.

\* G Holmdahl = G Bynke



- VI Bynke G, Håkanson R, Hörig J & Folkers K. 1984. Long-term administration of a substance P antagonist, (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP abolishes the response to ocular trauma. *Experientia*, in press.
- VII Bynke G, Håkanson R & Sundler F. 1984. Is corneal substance P necessary for corneal nociception? *Europ J Pharmacol*, in press.
- VIII Bynke G, Håkanson R, Hörig J & Folkers K. Substance P antagonists suppress ocular response to injury. A comparison of five antagonists. Submitted to *Experientia*.
- IX Bynke G, Wahlestedt C, Beding B, & Håkanson R. Pupillary hypersensitivity to substance P following prolonged treatment with tetrodotoxin or substance P antagonists. Submitted to *Europ J Pharmacol*.
- X Bynke G, Bruun A & Håkanson R. Sympathectomy enhances the substance P-mediated response to infrared irradiation of the rabbit iris. Submitted to *Experientia*.





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## INTRODUCTION

The eye responds to mechanical or chemical irritation by vasodilation in the conjunctiva and iris, increased intraocular pressure (IOP), miosis, and breakdown of the blood-aqueous barrier with subsequent leakage of protein into the aqueous humour. The general opinion is that the blood-aqueous barrier is located in the ciliary processes.

The importance of an intact sensory innervation for the ocular response to injury was first recognized by Bruce (1913), who observed that the conjunctival hyperaemia induced by nitrogen mustard was reduced after retrobulbar anaesthesia and abolished after section and subsequent degeneration of the ophthalmic nerve. The results suggest a local sensory reflex compatible with the antidromic sensory vasodilation in the skin (Bayliss 1901; Langley 1923). Perkins (1957) showed that the ocular response to stimulation of the trigeminal nerve is similar to that produced by local injury and resistant to atropine.

As a result of more recent studies of prostaglandins and prostaglandin inhibitors, the response to various noxious and irritating stimuli in the rabbit eye is considered to be mediated partly by a neurogenic pathway, and partly by prostaglandins (PGs), the relative contribution of the two pathways depending upon the type of stimulus (Neufeld et al. 1972; Cole & Unger 1973; Unger et al. 1974, 1975, 1977; Eakins 1977). Chemical irritants such as formaldehyde appear to act mainly via the neurogenic pathway, since the IOP response is reduced by prior treatment with local anaesthetics or by intraocular administration of the neuronal blocker tetrodotoxin and abolished by trigeminal denervation (Butler et al. 1979). The response to mechanical irritation, e.g. paracentesis of the anterior chamber, may be associated primarily with PGs, since it is greatly reduced by indomethacin (Cole & Unger 1973), an inhibitor of PG synthesis (Vane 1971). The response to laser irradiation of the iris is thought to be mediated by both pathways, since it is reduced by indomethacin and by local anaesthetics and abolished by the

combination of the two drugs (Unger et al. 1974, 1977). Also the breakdown of the blood-aqueous barrier by infrared irradiation is probably dependent on both pathways, since it is reduced after topical or retrobulbar anaesthesia (Dyster-Aas 1965) and after indomethacin treatment (Bengtsson et al. 1975). Recently, Butler & Hammond (1980) emphasized the key role of the neurogenic pathway in the response to all types of noxious stimuli, since trigeminal denervation abolishes the response not only to laser irradiation of the iris (Butler et al. 1980 b) but also to prostaglandin  $E_1$  ( $PGE_1$ ).

The identity of the mediator released upon antidromic nerve stimulation has for long been a matter of dispute. One of the candidates, the neuropeptide, substance P (SP), is known to be present in the central nervous system and in most peripheral organs (for a recent review, see Pernow 1983). SP is thought to be a neurotransmitter in the central part of a population of sensory neurones (Lembeck 1953; Amin, Crawford & Gaddum 1954; Hökfelt et al. 1975; Otsuka & Konishi 1975) and there is evidence that it acts as transmitter also at the peripheral ends (Hökfelt et al. 1975; Olgart et al. 1977; Bill et al. 1979).

There are several requirements which have to be met before accepting SP as a neurotransmitter in ocular sensory nerves. Firstly, SP has to be shown to be a neuronal constituent in the eye. Secondly, SP should be released upon stimulation of the trigeminal nerve. Thirdly, the effects of SP on the eye should mimic those of trigeminal nerve stimulation, and an antagonist of SP should block these effects.

Using radioimmunoassay, SP was demonstrated in the rabbit eye, particularly in the iris and the ciliary body, the optic nerve and the retina (Butler et al. 1980 a; Unger et al. 1981 b). In the anterior segment of the eye, SP-containing nerve fibres were found mainly in the pupillary sphincter and in the ciliary processes (Tervo et al. 1981; Stone et al. 1982; Tornqvist et al. 1982; Ehinger et al. 1983), and to a much lesser extent in the cornea (Tervo et al. 1981; Ehinger et al. 1983). These nerve fibres are thought to originate in the trigeminal ganglion (Miller et al 1981; Tervo et al. 1981, 1982; Keen et al. 1982). About

20-30% of the nerve cell bodies in the rabbit trigeminal ganglion are SP immunoreactive and the ophthalmic nerve also contains SP-positive nerve fibres (Hökfelt et al. 1975; Tervo et al. 1981). The trigeminal origin of the SP fibres in the anterior uvea is supported by other observations. Thus, Bill et al. (1979) showed that SP is released into the aqueous humour by electrical or mechanical stimulation of the trigeminal nerve, and Butler et al. (1980 a) found that diathermic destruction of the trigeminal ganglion is followed by reduced SP levels in the uvea.

Injection of SP into the eye produces miosis and increase of protein content in the aqueous humour (Bill et al. 1979; Stjernschantz et al. 1981). Six to eight days after diathermic destruction of the trigeminal nerve in the rabbit, the ocular hypertensive and miotic responses to intracameral administration of capsaicin, bradykinin and  $\text{PGE}_1$  are greatly reduced or abolished (Butler & Hammond 1980), whereas intracameral injection of SP still induces miosis. Therefore, it was proposed that the mediator released from sensory nerve endings by ocular irritants is SP (Zhang et al. 1982).

Capsaicin and SP antagonists are frequently used in SP research today. Capsaicin, the irritating agent of red pepper (see Nagy 1982), which stimulates certain sensory nerve endings and then makes them non-excitabile (Jancsó et al. 1967; Jancsó 1968), releases SP from central (Jessell et al. 1978; Yaksh et al. 1979; Gamse et al. 1981) as well as peripheral (Hayes & Tyers 1980) projections of sensory neurones. Interestingly, only chemical pain associated with C fibers is evoked and then abolished by capsaicin (Jancsó 1968; Szolcsányi 1977). In the rabbit, pre-treatment with capsaicin prevents the initial ocular hypertension induced by topically applied nitrogen mustard (Camras & Bito 1980). This supports the hypothesis that SP plays a role in the ocular response to chemical irritants.

Recently, Folkers et al. (1981) succeeded in developing SP antagonists by strategic amino acid substitutions in the SP molecule (see Rosell & Folkers 1982; Rosell et al. 1983) (Fig.1).

|                                               |     |                                   |               |
|-----------------------------------------------|-----|-----------------------------------|---------------|
| Arg - Pro - Lys - Pro - Gln - Gln - Phe       | Phe | Gly - Leu - Met - NH <sub>2</sub> | Substance P   |
| Pyr - Pro - Asp - Pro - Asn - Ala - Phe       | Tyr | Gly - Leu - Met - NH <sub>2</sub> | Uperolein     |
| Pyr - Ala - Asp - Pro - Asn - Lys - Phe       | Tyr | Gly - Leu - Met - NH <sub>2</sub> | Physalaemin   |
| Pyr - Asn - Pro - Asn - Arg - Phe             | Ile | Gly - Leu - Met - NH <sub>2</sub> | Phyllomedusin |
| Pyr - Pro - Ser - Lys - Asp - Ala - Phe       | Ile | Gly - Leu - Met - NH <sub>2</sub> | Eledoisin     |
| Asp - Val - Pro - Lys - Ser - Asp - Glu - Phe | Val | Gly - Leu - Met - NH <sub>2</sub> | Kassinin      |

#### SP antagonists

|              |                              |            |                             |
|--------------|------------------------------|------------|-----------------------------|
| Arg- D-Pro   | Lys - Pro - Gln - Gln- D-Trp | Phe- D-Trp | Leu - Met - NH <sub>2</sub> |
| D-Arg- D-Pro | Lys - Pro - Gln - Gln- D-Trp | Phe- D-Trp | Leu - Leu - NH <sub>2</sub> |
| D-Arg - Pro  | Lys - Pro - Gln - Gln- D-Trp | Phe- D-Trp | Leu - Leu - NH <sub>2</sub> |
|              | Arg - Gln- D-Trp             | Phe- D-Trp | Leu - Met - NH <sub>2</sub> |

Fig 1. Amino acid sequences of SP and SP-related tachykinins. Amino acids common to all tachykinins are boxed. Below are the amino acid sequences of a few SP antagonists to illustrate which substitutions generate antagonists.

The SP antagonists were found to block the action of SP in a specific and competitive manner (Folkers et al. 1981, 1984; Leander et al. 1981; Håkanson et al. 1982).

#### AIM OF THE PRESENT STUDY

The aim of the present study was to elucidate the role played by SP in the ocular response to injury, taking advantage of two powerful pharmacological tools, capsaicin and SP antagonists. The hypothesis to be tested was that SP is a neurotransmitter in a population of sensory nerve fibres in the eye and that neuronal SP is important for the response to injury. The study includes an attempt to assess the capacity of various SP antagonists for affecting the ocular response to local trauma.



## MATERIAL AND METHODS

### Animals

All experiments were performed on adult pigmented rabbits of both sexes and of mixed strain (1.0 - 3.5 kg). The animals were kept in the same environment for at least one week before being used for experiments. They were given a standard diet of pellets and water. The in vivo experiments, which constituted the main part of this study, were performed in the Department of Experimental Ophthalmology I. The in vitro studies were performed in the Department of Pharmacology.

### Measurement of the breakdown of the blood-aqueous barrier

Protein leakage into the anterior chamber was used as a parameter for quantifying the breakdown of the blood-aqueous barrier. The protein content can be measured by cannulating the anterior chamber, but since this procedure in itself may induce miosis and breakdown of the blood-aqueous barrier, an atraumatic photoelectrical method was used. By this method, the time course of the disruption of the blood-aqueous barrier can be followed in each individual.

The photoelectrical instrument (Bengtsson, Krakau & Öhman 1975) is a modification of a design presented by Dyster-Aas & Krakau (1963). The principle is illustrated in Fig. 2. A slit lamp produces a narrow beam of light (B). When this passes the anterior chamber, an aqueous flare (AF) is produced by scattering from large molecular aggregates in the aqueous humour. The slit lamp is rigidly connected with a system for measuring and observing the flare. A lens (L) forms an image of the flare and its surroundings in front of a photomultiplier tube (P), where a vibrating aperture (A) is placed. This moves with a frequency of 20 Hz, scanning over the flare. An AC-signal is produced by the multiplier tube, which is fed through a band pass filter ( $f = 40$  Hz), amplified, rectified and recorded. The flare density values, given in arbitrary units, are measured on paper records. By means of a beam splitter (S), part of the light reaches the examiner's eye (E), and enables the examiner to adjust the instrument to the

correct position for measuring. A glass sphere, occupying the same position as the rabbit's eye, is used for calibration purposes.

The aqueous flare is mainly caused by proteins. There is an almost linear positive correlation between the flare values and the protein concentration (Anjou & Krakau 1961; Dyster-Aas & Krakau 1964 a).

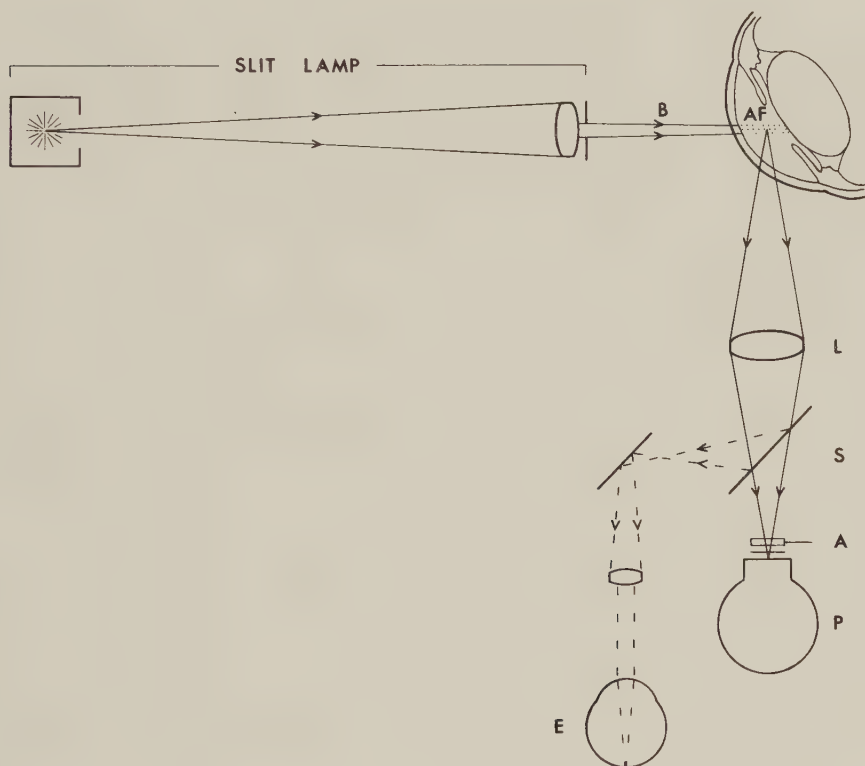


Fig. 2. The photoelectrical instrument  
(For explanation, see text).

### Pupil size

In most of the experiments, the changes of the pupillary diameter were recorded. The measurements were made during constant and uniform illumination using a clear plastic ruler, and were ex-

pressed either as the pupillary diameter or the pupillary constriction, i.e. the difference between the pupillary diameter before stimulation and the minimal diameter after stimulation. The measurements were made immediately or 5-15 min after the injury and then with 30 min intervals. It should be emphasized that small pupillary changes can be overlooked with this method.

#### Corneal sensitivity

The corneal sensitivity was tested by using a tactile and a chemical stimulus. The tactile stimulus consisted of cotton swabs soaked in water and gently applied to the cornea. The chemical stimulus was a drop of 0.12% capsaicin, applied to the eye. The sensitivity to this stimulus was tested with the wiping test (Szolcsányi & Jancsó-Gábor 1975), i.e. by counting the ensuing number of wipings with the forepaws.

#### Studies on isolated iris

The rabbit iris was excised, opened and mounted vertically on a Perspex holder in a 7 ml organ bath maintained at 33-35° C. One end was attached to a rigid support and the other one to a lever connected via a spring to a Grass FTO3 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Alternatively, isometric recording with a resting load of about 150 mg maintained during the experiments was used. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm and the electrodes were connected to a Grass S 4 C stimulator for field stimulation with square wave pulses (14-17 V over the electrodes, 5-20 Hz, 0.25 ms or 0.5 - 1 ms duration). The muscles were stimulated with trains of pulses with a resting period between each train. The mechanical activity of the muscles was recorded throughout the experiments. The bathing fluid was a modified Krebs solution, bubbled with a gas mixture of 7% CO<sub>2</sub> in O<sub>2</sub> providing a pH of 7.2-7.3. The preparations were allowed to equilibrate in the bath for 90 min before experimentation started.

#### Histological procedures

The cornea was dissected from the rabbit eyes immediately after death (intravenous injection of air). Whole mounts were prepared

by stretching the cornea onto chrome alum-coated glass slides which were then allowed to dry for a few minutes. Some of the whole mounts were fixed in Stefanini's fixative for 18 hours, rinsed in 10% sucrose, dehydrated and rehydrated repeatedly and then processed for the immunocytochemical demonstration of SP using a commercially available monoclonal SP antiserum (Immunonuclear, Stillwater, Okla., USA), and the indirect immunofluorescence technique described by Coons et al. (1955).

Other whole mounts were kept in a  $\text{Na}_2\text{SO}_4$  solution (24%) at  $37^\circ\text{C}$  over night. They were then incubated for five hours at room temperature in a solution of acetylthiocholine iodide for acetylcholinesterase staining according to the technique of Koelle (1963). The specificity of the staining was confirmed by using  $\text{N}_1\text{N}$ -diisopropylphosphorodiamide fluoride,  $4 \times 10^{-6}\text{ M}$ , to inhibit butyrylcholinesterase and 284 C 51 (1.5 bis allyldimethyl-ammonium-phenyl)pentan-3-one dibromide,  $10^{-5}\text{ M}$ , to inhibit acetylcholinesterase. The sections were counterstained in eosin and mounted in Permount<sup>R</sup>.

#### Aqueous flare provoking stimuli

Disruption of the blood-aqueous barrier was elicited by the following stimuli:

1. Infrared irradiation (IR) of the iris for 2 min (I, II, VI, VII, VIII, X)
2.  $\alpha$ -MSH\* (CIBA, Basel, Switzerland), given subcutaneously (20  $\mu\text{g}/\text{kg}$ ) (I)
3. Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) (Sigma, St Louis, Mo., USA), topically applied to the cornea (0.85-2.5  $\mu\text{g}$ ) (I,V)
4. Substance P (SP) (Beckman, Geneva, Switzerland), given intravitreally (0.3, 3, 30 or 300 pmoles) (II,III,IX,X)
5. Bradykinin (Beckman), given intravitreally (250 pmoles, 375 pmoles, 3.75 nmoles) (III,IV)
6. Capsaicin (Sigma), given retrobulbarly (0.15 ml 0.33%, 0.5 ml 1%) (I,IV,V,VII).

\*  $\alpha$ -MSH =  $\alpha$ -melanocyte stimulating hormone



The effect of the following substances or procedures on the response to subsequently applied aqueous flare provoking stimuli was studied:

1. Capsaicin, given retrobulbarly (0.5 ml 1%) (I,VII)
2. SP antagonists, given intravitreally or applied topically (see Table I)
3. Tetrodotoxin (Sigma); a neurotoxin obtained from the pufferfish, given intravitreally (5-10  $\mu$ g) (III,IV,V,VII,IX).
4. Oxibuprocaine (Apoteksbolaget, Sweden); a local anaesthetic, applied topically (50  $\mu$ l 0.4%) (VII).
5. Isopto-Atropine (Alcon, Fort Worth, Tex., USA); a muscarinic receptor antagonist applied topically (50  $\mu$ l 1%) (II,III)
6. Sympathectomy; known to enhance the ocular response to injury (Unger 1977) and to increase the SP content in the iris-ciliary body (Cole et al. 1983) (X).

The animals were anaesthetized with methohexital sodium (Brietal<sup>R</sup>, Lilly, Indianapolis, Ind., USA) when injected intravitreally or retrobulbarly and with pentobarbital (Mebumal<sup>R</sup>) during sympathectomy. No anaesthesia was needed during the rest of the experiments.

TABLE I

## SUBSTANCE P ANTAGONISTS USED IN THIS STUDY

| Code     | Sequence                                                          | Dose range<br>nmoles/app1 | Paper no                             |
|----------|-------------------------------------------------------------------|---------------------------|--------------------------------------|
| AP 2     | Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub> | 0.9-300                   | II, III, IV, V, VI, VII, VIII, IX, X |
| AP 4     | Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub>                   | 90-300                    | III, VIII, IX                        |
| AP 8     | Phe-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub>                   | 90                        | VIII                                 |
| AP 13    | Arg-Gln-D-Trp-Phe-D-Trp-Leu-Nle-NH <sub>2</sub>                   | 90                        | VIII                                 |
| Spantide | D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH <sub>2</sub> | 30-300                    | IV, VIII                             |

## RESULTS

### A. Intraocular effects of noxious stimuli

Substance P (SP). Intravitreal injection of SP produced a dose-dependent miosis but only a slight aqueous flare (II). Topical pretreatment with atropine affected neither the miosis nor the flare (II). Intravitreal injection of SP did not reduce the effect of a subsequent intravitreal injection of SP (II,III).

Capsaicin. Retrobulbar injection of a large dose of capsaicin (0.5 ml 1%) produced a prompt inflammatory reaction with conjunctival hyperaemia, chemosis, miosis (I,IV), and a strong aqueous flare response (AFR), which subsided gradually over a period of 1-2 days (I). Even the contralateral eye responded with a slight aqueous flare (I). Retrobulbar injection of a smaller dose of capsaicin (0.15 ml 0.33%) caused transient miosis (IV,V) and a moderate AFR, lasting for a few hours only (V). Repeated administration of capsaicin elicited a much reduced response (Bynke, unpublished observation).

Bradykinin. Intravitreal injection of a low dose of bradykinin (375 pmoles) caused miosis within 5-10 min (III,IV) and a moderate AFR, lasting for a couple of hours (III). Repeated administration of bradykinin (250 pmoles) 3-5 hours later elicited a much reduced response (III). Topical application of atropine before the injection of bradykinin did not prevent either the miotic response or the AFR (III).

Prostaglandin  $E_2$  ( $PGE_2$ ). Topical application of  $PGE_2$  (0.85  $\mu$ g, 2.5  $\mu$ g) caused moderate miosis and a dense aqueous flare (I,V).

$\alpha$ -MSH. Subcutaneous injection of  $\alpha$ -MSH (20  $\mu$ g/kg) produced AFR but no miosis (I). Anjou, Krakau & Stigmar (1961) found that systemic administration of a moderate dose of adrenocorticotrophic hormone (ACTH) to rabbits causes an increased aqueous flare in the anterior chamber. Later Dyster-Aas & Krakau (1964 b) showed that the flare provoking effect of ACTH is correlated with the melanocyte stimulating activity of ACTH. The disruptive effect of  $\alpha$ -MSH on the blood-aqueous barrier is not associated with increased prostaglandin synthesis, since it cannot be prevented by pretreatment with salicylates (Neufeld et al. 1972) or indome-

thacin (Bengtsson et al. 1975).

Infrared irradiation (IR). Infrared irradiation of the iris caused immediate miosis followed by AFR, reaching a maximum after about one hour (I,II,VI,VII,VIII,X). Compared with argon laser irradiation, IR (Dyster-Aas & Krakau 1964 a) represents a very mild trauma. It is thought to act partly via prostaglandins (Bengtsson et al. 1975), since the flare is inhibited by indomethacin.

## B. Modulation of the effects of noxious stimuli

Blockade of neuronal conduction. Intravitreal injection of tetrodotoxin (TTX), a well-known blocker of nervous conduction (Kao 1966), reduced both the AFR and the miotic response to topically applied  $\text{PGE}_2$  (0.85  $\mu\text{g}$ ) (V) and to IR of the iris (VII), but not to intravitreal injection of SP (30 pmoles) (III). TTX blocked the response to low doses of bradykinin or capsaicin (III,IV,V) but not to high doses (IV).

Capsaicin pretreatment. Pretreatment with retrobulbar injections of capsaicin reduced the AFR to subsequent IR of the iris, to subcutaneously administered  $\alpha$ -MSH, and to topically applied  $\text{PGE}_2$  (2.5  $\mu\text{g}$ ) (I). In the case of IR and  $\alpha$ -MSH, the reduced responsiveness was manifest for several weeks after capsaicin pretreatment, involving first the capsaicin-treated eye, but later also the contralateral eye. After 2-3 months, the AFR was normal in both eyes. In the case of  $\text{PGE}_2$ , the responsiveness was reduced for a shorter time; after 3 weeks the response was normal in both eyes.

SP antagonists. Intravitreal injection of low doses of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP (0.9 nmoles, 3 nmoles, 9 nmoles) had no effects on the eye (II). Doses of 30 and 90 nmoles elicited a moderate, rather long-lasting miosis but no AFR (II). Pretreatment with intravitreal injections of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP reduced both the miosis and the AFR to subsequent IR of the iris in a dose-dependent manner (II) and also the miotic response and the AFR to intravitreal injection of SP (II).



Topical application of (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP had no effect on the eye in itself, but reduced the AFR to subsequent IR in a dose-dependent manner (II,VI,VIII,X). The effect lasted for 3 but not for 5 hours (VI). Topical application of (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP, or (Arg<sup>5</sup>,D-Trp<sup>7,9</sup>)-SP or (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP reduced the miotic response and the AFR to a low dose of bradykinin (375 pmoles) (III,IV). Topical application of (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP reduced the miosis and AFR induced by a low dose of capsaicin (150  $\mu$ l 0.33%)(IV,V) and a low dose of PGE<sub>2</sub> (0.85  $\mu$ g) (V). A series of previously characterized SP antagonists (Table I) were compared for potency with respect to suppression of the ocular response to IR of the iris. (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP and (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP were the most potent ones (VIII). The difference between these two antagonists with respect to their ability to reduce the AFR was small.

Long-term administration (2-3 months) of a large dose of (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP by the topical route abolished the AFR to IR of the iris (VI) and inhibited the miosis caused by a large dose of SP during ongoing treatment (IX). By a small dose of SP, the miosis was inhibited both in the treated and in the contralateral eye as compared with the miosis elicited by the same dose of SP in the control group (IX). Two days after termination of treatment, IR of the iris still evoked a reduced response (VI). Another two days later IR provoked a normal AFR (VI).

Sympathectomy. One month after sympathectomy the AFR to IR of the iris was greatly enhanced. This enhancement could be blocked by pretreatment with (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (X). Sympathectomy did not affect the miotic response to SP (X).

#### Studies on the isolated iris

The pupillary sphincter contracted slowly after addition of SP to the bath, with a maximum after 120-150 s (III). The bradykinin- and capsaicin-induced contraction was even slower at onset (III,IV). Repeated administration of SP did not induce any change in the responses, whereas the response to bradykinin or capsaicin displayed marked tachyphylaxis (III,IV). Exposure to capsaicin reduced the capacity of the pupillary sphincter to respond to subsequent application of bradykinin and vice versa ("cross-tachyphylaxis") (IV). A preliminary study suggested that the

contractile response to bradykinin was reduced by pretreatment with TTX (III). However, a subsequent study revealed that TTX did not affect the contractile response to either SP, bradykinin or capsaicin (IV). The contractile responses to SP, bradykinin and capsaicin were greatly reduced by pretreatment with (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP, (Arg<sup>5</sup>,D-Trp<sup>7,9</sup>)-SP or (D-Arg<sup>7</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP (III,IV). The pupillary sphincter responded to electric stimulation with a rapid twitch followed by a slow contraction, reaching a maximum 20-30 s after cessation of stimulation (IV). Atropine blocked the twitch but was without effect on the slow contraction (IV). The atropine-resistant contraction could be abolished by SP antagonists, indicating that the non-cholinergic contraction is mediated by SP (IV). After exposure to bradykinin or capsaicin the non-cholinergic contraction was reduced (IV).

#### Hypersensitivity to substance P.

Repeated intravitreal injections of TTX caused pupillary hypersensitivity to SP, pilocarpine and neosynephrine (IX). Long-term administration (2 months) by the topical route of large doses of SP antagonists provoked pupillary hypersensitivity to SP but not to pilocarpine (IX). In vitro, the slow contraction produced by electric stimulation became much more predominant both in the treated and in the contralateral sphincter, suggesting development of hypersensitivity to SP (IX).

#### Corneal sensitivity.

No effect on corneal sensitivity was observed after long-term administration of large amounts of topically applied (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (VI,VII). As expected, the local anaesthetic oxibuprocaine and TTX made the cornea insensitive to tactile as well as chemical stimuli (VII). Retrobulbar injections of capsaicin had no obvious effect on the sensitivity to tactile stimuli but the sensitivity to chemical stimuli was transiently reduced (VII). A few varicose SP fibres could be demonstrated in the cornea by immunocytochemistry (VII). By contrast, acetylcholinesterase staining revealed a dense network of nerve fibres (VII). Forty-eight hours after capsaicin pretreatment (retrobulbar injection) no SP fibres could be detected in the cornea while the cholinesterase-positive nerve fibres seemed unaffected (VII).

## DISCUSSION

The ocular response to local injury is dependent upon neurogenic mechanisms, probably involving orthodromic as well as antidromic impulse traffic in sensory nerve terminals (Fig. 3).

Recently, interest has focussed on SP as a possible mediator in sensory nerve fibres. The following observations support the view that SP is a neurotransmitter in the uvea:

1. SP exists in sensory nerve fibres in the uvea (Tervo et al. 1981; Stone et al. 1982; Tornqvist et al. 1982; Ehinger et al. 1983).
2. SP is released by electric stimulation of the trigeminal nerve (Bill et al. 1979) and by certain irritants, such as capsaicin (Mandahl et al. 1984).
3. SP induces effects in the eye which mimic those seen after application of physical or chemical irritants (Bill et al. 1979; Stjernschantz et al. 1981; II,III,IX).
4. SP antagonists counteract the intraocular responses not only to SP but also to other physical and chemical irritants (II,III,IV,V). This effect does not seem to be related to any general neurotoxic action of any of these agents, since a) the effect is reversible (VI), and, b) these agents do not interfere with cholinergic (IX), or nociceptive nerve stimulation (VII).

The results of the present study support the hypothesis that substance P (SP) or related peptides play a key role in the ocular response to various types of noxious stimuli. Pretreatment with capsaicin, an SP depleting agent, prevented disruption of the blood-aqueous barrier caused by IR of the iris, subcutaneously administered  $\alpha$ -MSH or topically applied PGE<sub>2</sub> (I). These results are compatible with the findings by Camras & Bito (1980) that capsaicin pretreatment inhibits the initial ocular hypertension caused by nitrogen mustard. The lack of responsiveness is probably due to depletion of SP from sensory nerve fibres in the ciliary processes. Retrobulbar injection of capsaicin induces miosis and breakdown of the blood-aqueous barrier (I,V). The miotic and the aqueous flare responses to a low dose of capsaicin

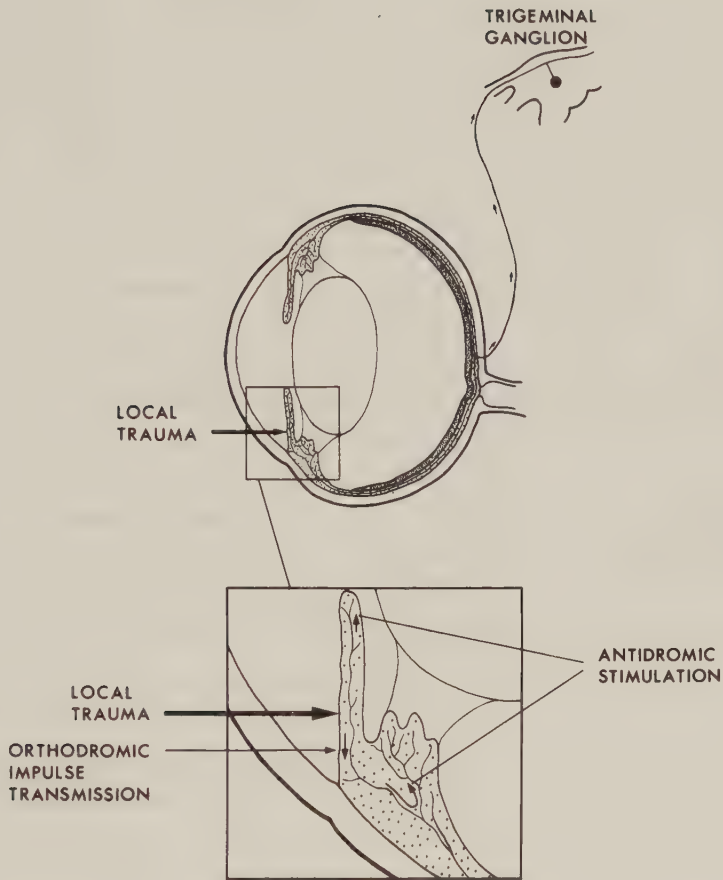


Fig. 3. A schematic drawing, illustrating the proposed neurogenic pathways in the ocular response to local physical or chemical injury. As a result of the trauma orthodromic and antidromic impulse transmission is generated in sensory nerve fibres, originating in the trigeminal ganglion. Antidromic impulse transmission is supposed to be responsible for much of the local response to trauma. Recent observations suggest an important role for substance P in the mediation of the neurogenic response.



can be reduced either by TTX or by (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (IV,V) and are thus dependent on SP-containing neurones. Consistently, Mandahl & Bill (1981, 1983) observed that the miotic response to a low dose of capsaicin was blocked by TTX or by (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP. However, the contractile response of the isolated pupillary sphincter to capsaicin was resistant to TTX but reduced by (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP (IV). The results obtained with bradykinin were similar. The miotic response and the AFR caused by a low dose of bradykinin could be reduced by SP antagonists or by TTX (III,IV). However, the contractile response to bradykinin of the isolated pupillary sphincter could be reduced by SP antagonists only but not by TTX (IV). This indicates the existence of bradykinin receptors on smooth muscle and on sensory nerve endings. The effects of capsaicin and bradykinin on the eye seem to depend upon the presence of functioning sensory nerves and on release of a neurogenic mediator, probably SP. The direct effect of capsaicin and bradykinin on the sensory nerve endings is resistant to TTX. The impulse traffic generated by exposure to capsaicin and bradykinin travels orthodromically and antidromically (axon reflex) in the eye to cause further release of mediator from sensory nerve endings in the ciliary processes and in the pupillary sphincter. This effect is sensitive to TTX.

Even the miosis and AFR to PGE<sub>2</sub> were reduced by (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP or TTX (V). Thus, the effect of PGE<sub>2</sub> is at least partly mediated by sensory nerves and requires nervous conduction. Similar results were obtained by Mandahl & Bill (1983), who reported a reduction of the miosis and the aqueous protein content induced by PGE<sub>1</sub> after pretreatment with (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP.

The aqueous flare response to infrared irradiation of the iris, previously shown to be mediated partly by PGs (Bengtsson, Krakau & Öhman 1975), was also reduced by pretreatment with (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (II,VI). The AFR to IR of the iris was also reduced by TTX or by prolonged treatment with the local anaesthetic oxibuprocaine (VII), compatible with the ocular response to laser irradiation of the iris (Unger et al. 1977). This suggests that IR stimulates sensory nerve terminals in the pupillary sphincter and in the ciliary processes to release SP. From

these terminals impulses pass orthodromically and antidromically by axon reflex to release further mediator from the terminals.

The best SP antagonists in reducing the AFR to IR of the iris were found to be (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP and (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP (VIII). These antagonists were almost equally potent in reducing the AFR to IR. This was surprising in view of the fact that (D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>)-SP is ten times more potent than (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP in vitro (Folkers et al. 1984). Long-term administration of (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP abolished the AFR to IR of the iris (VI), which emphasizes the importance of the neurogenic pathway in the ocular response to trauma.

The miotic response and the AFR to SP was reduced by (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (II) but not by TTX (III; Bynke et al. 1984), suggesting that SP acts directly on the pupillary sphincter and on the ciliary processes. After long-term administration of SP antagonists, the miotic response to a small dose of SP was inhibited both in the treated and untreated eye (IX), suggesting that with time the SP antagonist passes over to the contralateral eye in amounts sufficient to cause effects. The miotic response to a large dose of SP was inhibited in the treated but not in the contralateral eye (IX). Following prolonged treatment with TTX or long-term treatment with SP antagonists, pupillary hypersensitivity to SP was induced (IX).

About one month after sympathectomy, the AFR to IR of the iris was greatly enhanced (X); this enhancement could be blocked by pretreatment with (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP (X). It was previously shown that the ocular response to laser irradiation of the iris increases after sympathectomy (Unger 1977; Unger et al. 1981 a) and also that the SP level in the uvea is increased (Cole et al. 1983). This suggests a functional interaction between the sympathetic system and the sensory system and lends further support to the view that SP plays an important role in the ocular response to injury.

The cornea is richly supplied with sensory nerve fibres supposed to derive from the trigeminal nerve (Zander & Weddell 1951), but only a minor proportion of this supply consists of SP fibres (Tervo et al. 1981; Ehinger et al. 1983; VII). Treatment

with oxibuprocaine or TTX abolished the corneal sensitivity to tactile and chemical stimuli but pretreatment with capsaicin or (D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>)-SP did not (VII). Consequently, it is suggested that the neurogenic mechanisms involved in corneal nociception do not depend upon corneal SP (VII).

#### GENERAL SUMMARY

The results of the present papers strongly suggest that substance P (SP) or a related peptide is a neuronal mediator of the ocular response to local injury, because:

1. SP in itself produces miosis and aqueous flare response (AFR).
2. Pretreatment with capsaicin, an SP depleting agent, prevents the AFR to a subsequent trauma such as infrared irradiation (IR) of the iris, subcutaneously administered  $\alpha$ -MSH and topically applied prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), suggesting that these stimuli act on sensory nerve endings in the ciliary processes to release a mediator, probably SP, which causes disruption of the blood-aqueous barrier.
3. Pretreatment with the neuronal blocker TTX reduces the miosis and the AFR to IR of the iris, PGE<sub>2</sub>, bradykinin, and capsaicin, but not to SP, indicating that the actions of SP are not mediated by nerves.
4. Pretreatment with SP antagonists reduces the miosis and the AFR to IR of the iris, PGE<sub>2</sub>, bradykinin, capsaicin and SP.
5. Long-term treatment with an SP antagonist abolishes the AFR to IR of the iris, emphasizing the importance of the neurogenic pathway in the ocular response to injury.
6. Pupillary hypersensitivity to SP occurs after prolonged treatment with TTX or an SP antagonist.
7. The enhanced intraocular response to local injury following sympathectomy may reflect the previously described increase in the SP level in the uvea, since this enhancement is abolished by pretreatment with an SP antagonist.
8. SP antagonists do not seem to be generally neurotoxic, since they have no effect on corneal sensitivity or on cholinergic neurotransmission in the isolated pupillary sphincter. Furthermore, the effects of SP antagonists are reversible.

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# Capsaicin Pretreatment Prevents Disruption of the Blood-aqueous Barrier in the Rabbit Eye

Gunnel Bynke

Capsaicin, the irritating agent of red pepper, produces ocular inflammation through a neurogenic mechanism. The present study is concerned with the long-term effects of capsaicin pretreatment on the capacity of the eye to respond to different inflammatory stimuli. Following retrobulbar injection of capsaicin to rabbits the aqueous flare response induced by subsequent infrared irradiation (IR) of the iris, subcutaneously administered  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) and exogenously administered prostaglandin  $E_2$  ( $PGE_2$ ) was reduced greatly. In the case of IR and  $\alpha$ -MSH the reduced responsiveness was manifest for several weeks after capsaicin pretreatment, involving first the capsaicin-treated eye, but later also the contralateral control eye. After 2–3 months the aqueous flare response was normal in both eyes. In the case of  $PGE_2$  the responsiveness was reduced for a shorter time; after 3 weeks the response was normal in both eyes. The results indicate that all three stimuli tested are at least partly dependent upon an intact sensory innervation to disrupt the blood-aqueous barrier, but that the mechanism of action of  $PGE_2$  is different from that of IR and  $\alpha$ -MSH. *Invest Ophthalmol Vis Sci* 24:744–748, 1983

The inflammatory response to trauma of the eye is characterized by initial ocular hypertension, miosis, and breakdown of the blood-aqueous barrier. It is at least partly mediated by antidromic stimulation of sensory nerve fibers, since trigeminal denervation or treatment with the neuronal blocking agent tetrodotoxin prevents or greatly reduces the response.<sup>1</sup> In the rabbit eye, electrical or mechanical stimulation of the trigeminal nerve causes miosis and other signs of inflammation.<sup>2</sup> Prostaglandins (PGs) seem to be involved in the inflammatory response to anterior chamber paracentesis and laser irradiation of the iris, since aspirin treatment prevents inflammation.<sup>3</sup> However, they do not appear to be involved in the response to trigeminal nerve stimulation or in the initial response to nitrogen mustard, both of which require an intact sensory neural pathway to disrupt the blood-aqueous barrier.<sup>4</sup>

Retrobulbar injection of capsaicin, the irritating agent of red pepper, produces ocular inflammation.<sup>5</sup> After normalization, the capsaicin-treated eye is insensitive to the initial hypertensive effect of nitrogen mustard.<sup>5</sup> The desensitizing effect of capsaicin has been attributed to its ability to deplete sensory neu-

rons of substance P (SP).<sup>5,6</sup> SP has been proposed to act as a mediator of the inflammatory response,<sup>7</sup> since it is thought to be a neurotransmitter in sensory afferents<sup>8</sup> and since exogenous SP is capable of eliciting responses characteristic of inflammation.<sup>9</sup> Furthermore, stimulation of the trigeminal nerve has been shown to release SP into the aqueous humor,<sup>9</sup> whereas trigeminal denervation reduces the SP content of the iris-ciliary body<sup>10</sup> and prevents the inflammatory response.<sup>10</sup>

The present report describes the effect of capsaicin pretreatment on the response to various blood-aqueous barrier disrupting agents: (1) infrared irradiation (IR) of the iris, thought to act partly via prostaglandins,<sup>11</sup> (2)  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH), which acts independently of prostaglandins,<sup>3,11</sup> and (3)  $PGE_2$ , which seems to be at least partly dependent upon neural activity to cause inflammation.<sup>12</sup>

## Materials and Methods

Adult pigmented rabbits (1.5–3 kg) of mixed strains were used. A 1% capsaicin solution was prepared by dissolving 100 mg of capsaicin (Sigma, St. Louis, MO) in 150  $\mu$ l of 99.5% ethanol; then 850  $\mu$ l of Tween 80 was added, followed by 9 ml of saline.<sup>5</sup> Retrobulbar injection of 0.5 ml of 1% capsaicin to the left eye was given as described by Camras and Bito.<sup>5</sup> Control animals were instead given the same volume of the vehicle.

$PGE_2$  (Leo, Helsingborg, Sweden) was dissolved in

From the Department of Experimental Ophthalmology, University Eye Clinic, Lund, Sweden.

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Reprint requests: Gunnel Bynke, MD, Department of Experimental Ophthalmology, University Eye Clinic, S-221 85 Lund, Sweden.

ethanol (10 mg/ml) and saline was added to give a solution containing 0.5 mg/ml.  $\alpha$ -MSH (Ciba, Basel, Switzerland) was dissolved in saline (100  $\mu$ g/ml). Metohexital sodium (Brietal<sup>R</sup>, Lilly, Indiana, USA) (10 mg/ml) was used for anesthesia. The animals were given Brietal (5 mg/kg body weight) when injected with capsaicin. No anesthesia was administered during the rest of the experiments.

The blood-aqueous barrier was disrupted by the following methods: 1) IR of the iris for 2 min<sup>13</sup>, 2) subcutaneous injection of  $\alpha$ -MSH (20  $\mu$ g/kg body weight), 3) topical application of PGE<sub>2</sub> (2.5  $\mu$ g) onto the cornea. These doses of  $\alpha$ -MSH and PGE<sub>2</sub> have previously been found to give an aqueous flare response.<sup>14</sup>

The course of the barrier damage was followed by measuring photoelectrically<sup>11</sup> the aqueous flare response every 30 min. A correlation between the density of the aqueous flare, expressed in arbitrary units with reference to a standard, and the protein content of the aqueous humor has been demonstrated previously.<sup>15</sup>

The corneal reflexes were tested by the use of wetted cotton swabs.

Analysis of variance (one-way and two-way) was used for calculating the significance of difference between treated and control eyes and between separate occasions in the same experimental group.

## Results

### Acute Effects of Capsaicin

In the treated eyes capsaicin produced a prompt inflammatory reaction with conjunctival hyperemia, chemosis, miosis, and a dense aqueous flare, which subsided gradually over a period of 1–2 days (Fig. 1). Even the contralateral eyes responded with a slight aqueous flare. The difference between the eyes was highly significant ( $P < 0.001$ ).

The pupillary light reflexes and the corneal reflexes were unaffected by capsaicin.

### Induction of Aqueous Flare after Capsaicin Pretreatment

When the aqueous flare response to capsaicin had subsided, attempts were made to disrupt the blood-aqueous barrier by IR,  $\alpha$ -MSH, and PGE<sub>2</sub>.

**IR:** Two to 4 days after pretreatment with capsaicin six rabbits were subjected to IR of both irides (Fig. 2A). The aqueous flare response was greatly reduced in the capsaicin-treated as compared with the contralateral eyes ( $P < 0.001$ ). Six weeks later three of these rabbits were again subjected to IR of both eyes (Fig. 2B). As compared with the corresponding eyes

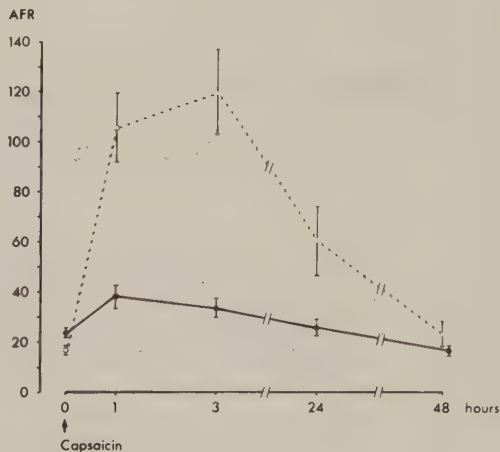


Fig. 1. Aqueous flare response (AFR) to retrobulbar injection of capsaicin of the left eye (dashed curve). Right eye (filled curve) served as control. Ordinate: mean flare density (arbitrary units)  $\pm$  SE (ten rabbits).

in Figure 2A the response was now reduced also in the contralateral eyes ( $P < 0.05$ ) and inhibited to the same degree in the capsaicin-treated eyes ( $P > 0.05$ ). There was no longer any difference between the capsaicin-treated and the contralateral eyes ( $P > 0.05$ ). After 3½ months the procedure was repeated in the same three animals (Fig. 2C). Now the response was no longer inhibited. Thus, as compared with the corresponding eyes in Figure 2A, it had increased in the capsaicin-treated eyes ( $P < 0.001$ ) and was back to the initial values in the contralateral eyes ( $P > 0.05$ ). There was no significant difference between the capsaicin-treated and the contralateral eyes ( $P > 0.05$ ).

**$\alpha$ -MSH:** Two to 3 days after capsaicin pretreatment of the left eyes  $\alpha$ -MSH was injected subcutaneously in five rabbits (Fig. 3A). The aqueous flare response was reduced greatly in the capsaicin-treated eyes as compared with in the contralateral eyes ( $P < 0.001$ ). Six weeks later three of these animals were again given  $\alpha$ -MSH (Fig. 3B). As compared with the corresponding eyes in Figure 3A, the response was now reduced also in the contralateral eyes ( $P < 0.001$ ) and inhibited to the same degree in the capsaicin-treated eyes ( $P > 0.05$ ). There was no longer any difference between the capsaicin-treated and the contralateral eyes ( $P > 0.05$ ). After 2½ months the procedure was repeated in the same three rabbits. Now the aqueous flare response was normal in both eyes (not shown in Fig. 3).

**PGE<sub>2</sub>:** Two days after capsaicin pretreatment of the left eyes PGE<sub>2</sub> was applied topically to both eyes

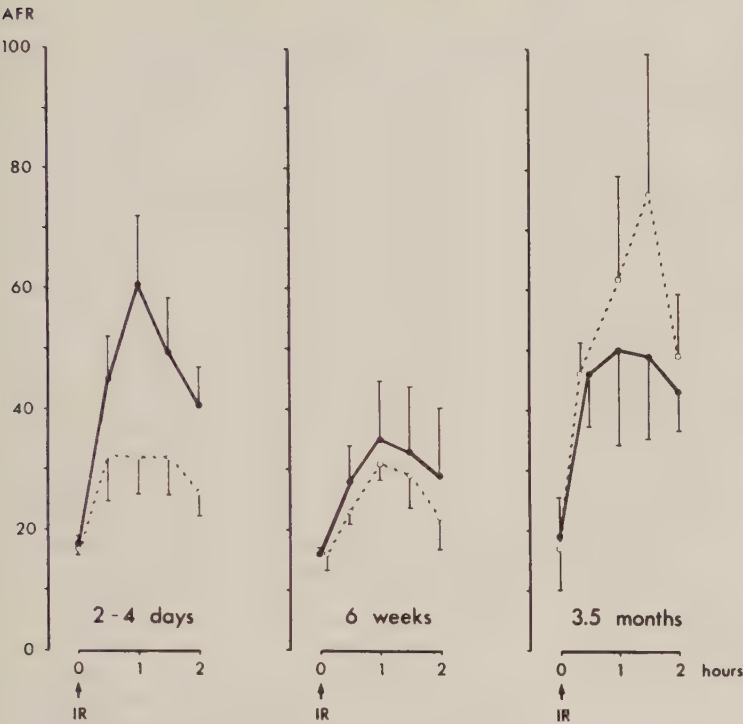


Fig. 2. Aqueous flare response (AFR) to infrared irradiation (IR) of the iris of both eyes 2-4 days (A, n = 6), 6 weeks (B, n = 3), and 3½ months (C, n = 3) after pretreatment with capsaicin of the left eye (dashed curve). Right eye (filled curve) served as control. Ordinate: mean flare density (arbitrary units) ± SE.

of five rabbits (Fig. 4A). The aqueous flare response was reduced greatly in the capsaicin-treated eyes as compared with the contralateral eyes ( $P < 0.001$ ). Three weeks later the same procedure was repeated

in all five animals (Fig. 4B). Now the response was no longer inhibited. Thus, as compared with the corresponding eyes in Figure 4A, it had increased in the capsaicin-treated eyes ( $P < 0.001$ ) and was unaltered

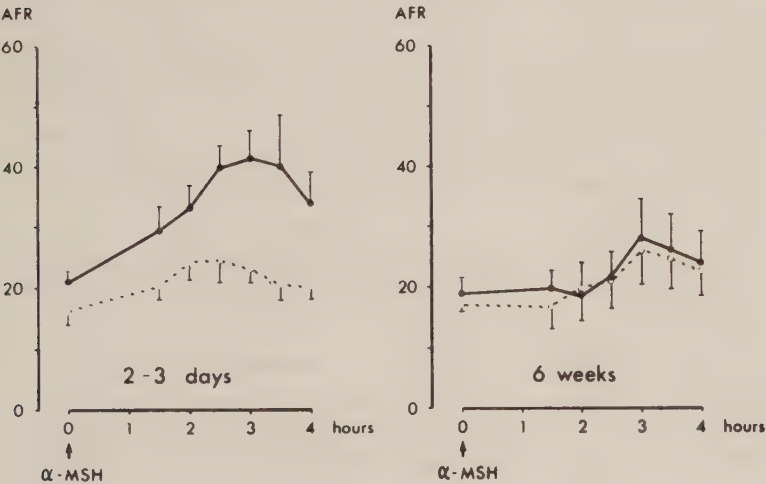
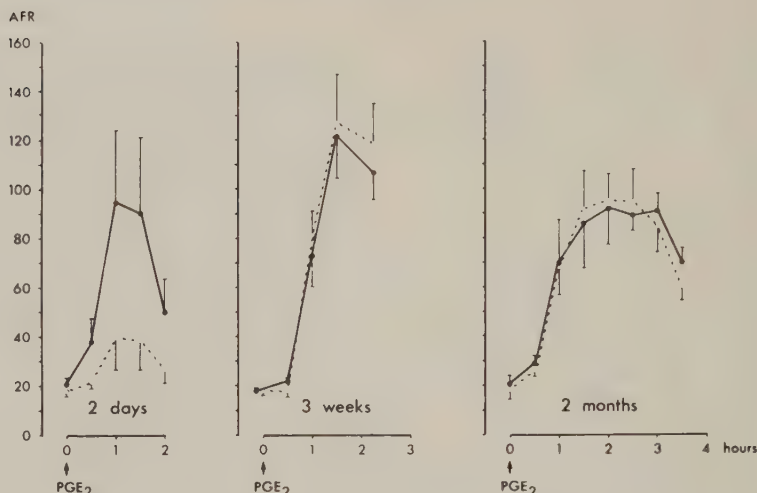


Fig. 3. Aqueous flare response (AFR) to subcutaneous injection of  $\alpha$ -MSH 2-3 days (A, n = 5) and 6 weeks (B, n = 3) after pretreatment with capsaicin of the left eye (dashed curve). Right eye (filled curve) served as control. Ordinate: mean flare density (arbitrary units) ± SE.

**Fig. 4.** Aqueous flare response (AFR) to topically applied PGE<sub>2</sub> 2 days (A, *n* = 5), 3 weeks (B, *n* = 5), and 2 months (C, *n* = 5) after pretreatment with capsaicin of the left eye (dashed curve). Right eye (filled curve) served as control. Ordinate: mean flare density (arbitrary units)  $\pm$  SE.



in the contralateral eyes ( $P > 0.05$ ). There was no longer any difference between the capsaicin-treated and the contralateral eyes ( $P > 0.05$ ). After 2 months the procedure was repeated in the same five animals (Fig. 4C). The aqueous flare response was normal in both eyes. Thus, there was no significant difference between the corresponding eyes in Figure 4C as compared with the corresponding eyes in Figure 4B ( $P > 0.05$ ), and there was no difference between the capsaicin-treated and the contralateral eyes ( $P > 0.05$ ).

At no stage did pretreatment with the vehicle (control animals) interfere with the aqueous flare response to IR,  $\alpha$ -MSH and PGE<sub>2</sub>.

### Discussion

Retrobulbar injection of capsaicin produced the expected inflammatory response. The fact that the response of the uvea to capsaicin is prevented or greatly reduced by trigeminal denervation<sup>16</sup> or by tetrodotoxin pretreatment<sup>12</sup> suggests that it depends upon an intact trigeminal nerve.

Pretreatment with capsaicin reduced the aqueous flare induced a few days later by IR,  $\alpha$ -MSH and PGE<sub>2</sub>, suggesting that these agents depend upon an intact sensory innervation to disrupt the blood-aqueous barrier. The inhibition of the response to IR and  $\alpha$ -MSH persisted for 6 weeks; at this stage the response was reduced also in the contralateral eye. The inhibitory effect of capsaicin was reversible and could not be demonstrated after 2–3 months. Contrary to the effect of IR and  $\alpha$ -MSH, the aqueous flare response to PGE<sub>2</sub> was normal already 3 weeks after capsaicin. This was surprising in view of the fact that

the responses to IR and  $\alpha$ -MSH at this stage were greatly reduced. Possibly, the nature of the neural involvement in the response to PGE<sub>2</sub> differs from that of the other inflammatory stimuli.

Capsaicin is thought to act on sensory nerve fibers by releasing SP. From this point of view, the lack of response to inflammatory stimuli following capsaicin pretreatment can be explained by degeneration of SP containing trigeminal nerve fibers, which are required for the neurogenic mediation of the inflammatory response. This concept is also consistent with the recent finding that specific SP antagonists inhibit the inflammatory response to IR<sup>17</sup> and bradykinin (Bynke et al, to be published). However, it should be noted that exogenous SP does not completely reproduce the inflammatory response to ocular trauma, in particular with respect to the relative inefficiency of SP in causing aqueous flare.<sup>17</sup> Perhaps SP is not the only neurogenic mediator of inflammation.

**Key words:** experimental ocular inflammation, blood-aqueous barrier, capsaicin, infrared irradiation,  $\alpha$ -melanocyte stimulating hormone, prostaglandin E<sub>2</sub>

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## **A Substance P Antagonist, [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP, Inhibits Inflammatory Responses in the Rabbit Eye**

G. Holmdahl, R. Håkanson, S. Leander, S. Rosell, K. Folkers and F. Sundler

# A Substance P Antagonist, [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP, Inhibits Inflammatory Responses in the Rabbit Eye

**Abstract.** Neurogenic factors released by antidromic nerve stimulation are thought to be in part responsible for the vasodilation and breakdown of the blood-aqueous barrier that follows trauma to the eye. Substance P is one candidate for the mediation of the inflammatory response since it is thought to be a neurotransmitter in sensory afferents and since exogenous substance P is capable of eliciting a response characteristic of inflammation. In rabbits, intravitreal or topical application onto the eye of a specific substance P antagonist, [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP, inhibited not only the irritant effects of exogenous substance P but also the inflammatory response to a standardized trauma (infrared irradiation of the iris). These observations suggest that substance P, or a related peptide, is a neurogenic mediator of the inflammatory response in the eye.

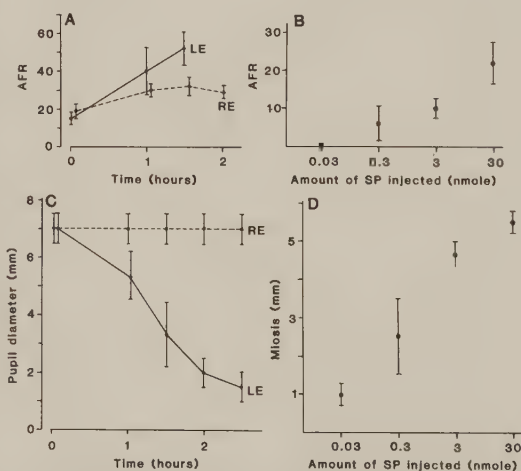
The inflammatory response to trauma in the eye, characterized by constriction of the pupil (miosis), hyperemia, and breakdown of the blood-aqueous barrier, is thought to involve antidromic stimulation of sensory nerve fibers since treatment with the neuronal blocking agent tetrodotoxin or trigeminal denervation prevents or greatly reduces the response (1, 2). Studies on skin suggest that both vasodilation and plasma extravasation results from local release of substance P (SP) by antidromic nerve stimulation of sensory nerve endings (3). It has been proposed that SP acts as a neurogenic mediator of the inflammatory response (3) since it is thought to be a neurotransmitter in sensory afferents (4) and since exogenous SP is capable of eliciting responses characteristic of inflammation (3, 5). Experiments with capsaicin, the irritating agent of red pepper, support this hypothesis. Capsaicin seems to release SP from sensory afferents, causing permanent damage with concomitant reduction in the SP levels (6, 7). Retrobulbar injection of capsaicin initially causes a strong inflammatory response which subsides slowly (8, 9); the response to a subsequent trauma, but not that to exogenous SP, is greatly inhibited and this inhibition seems to be long-lasting (9). That the response of the uvea to capsaicin depends on an intact trigeminal nerve is suggested by the fact that it is prevented or greatly reduced by denervation (10) or prior treatment with tetrodotoxin (11). The SP-containing nerve fibers in the anterior uvea are associated with iris smooth muscle and ciliary processes (12, 13). Intracameral (into the anterior eye chamber) injection of SP causes miosis and breakdown of the blood-aqueous barrier, thereby mimicking the response to trauma (5).

Among a series of SP analogs that has been synthesized (14, 15), one, [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP, is a weak agonist and a potent, specific, competitive antagonist of SP (15, 16). The isolated rabbit iris

sphincter pupillae muscle responds to electrical nerve stimulation with a contraction; neither adrenergic nor cholinergic blockade abolishes this response. The SP antagonist, however, prevents the nerve-mediated contraction, suggesting that SP is the neurotransmitter involved (16). We have further examined the role of SP in the inflammatory process by analyzing the effects of [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP on the response of the rabbit eye to a standardized trauma.

Adult pigmented rabbits (1.5 to 3 kg) of mixed strain were used. They were anesthetized with methohexital sodium (Brietal, Lilly; 5 mg/kg) when given injections into the eyes. No anesthesia was administered during the rest of the experiments. The blood-aqueous barrier of the eye was disrupted by infrared irradiation of the iris for 2 minutes (17), or intravitreal injection of SP. The time course of the barrier damage was followed by photoelectric measurement (18) of the aqueous flare response (AFR)

in the intact eye. This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and protein concentration has been established (19). The results are expressed in arbitrary units with reference to a standard. The AFR and pupillary diameter were measured every 30 minutes (if not otherwise stated). The corneal sensitivity was tested at the same time intervals by the use of wetted cotton swabs. Synthetic SP (Beckman, Geneva, Switzerland) and [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP were dissolved in 0.9 percent saline to give stock solutions with concentrations of  $4 \times 10^{-5}M$  and  $1.7 \times 10^{-3}M$ , respectively. The stock solutions of SP and [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP were serially diluted so that the volume injected was the same for the different doses (9  $\mu$ l for SP and 60  $\mu$ l for the SP analog). The solutions were injected into the corpus vitreum of the left eye unless otherwise stated. The corresponding volume of 0.9 percent saline was injected into the right eye (control eye). Intravitreal injections were given by means of Hamilton precision syringes 3 to 4 mm posterior to the limbus. The SP analog was injected 3 to 4 hours before irradiation of the iris or injection of SP, and the inflammatory response was followed for another 2 to 3 hours. In one series of experiments 60  $\mu$ l of atropine (Isopto-Atropin, Alcon; 1 percent) was applied topically onto the cornea 1 hour before irradiation of the iris or injection of SP. In a final series of experiments [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP was applied topically onto the cornea in a volume of 60  $\mu$ l, 1 hour



**Fig. 1.** (A and B) The AFR and (C and D) the miotic response to intravitreal injection of 30 nmole of SP into the left eye (LE) and 0.9 percent saline into the right eye (RE). The difference between the left and right eye was not significant with respect to AFR and highly significant ( $P < .001$ ) with respect to pupil diameter. (B and D) Dose-response curves showing (B) the maximum AFR (increase over starting value) of the SP-treated eye minus that of the control eye and (D) the decrease in pupil diameter (miosis) (D) after

intravitreal injection of SP. The correlation coefficient ( $r$ ) for AFR versus the amount of SP was .706 ( $P < .05$ ) and for miosis .805 ( $P < .01$ ). Three rabbits in each dose group were examined; vertical lines give the standard error of the mean.

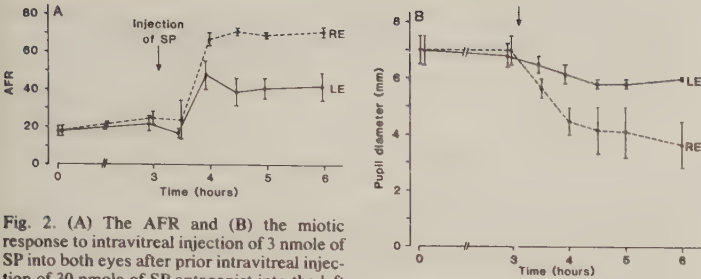


Fig. 2. (A) The AFR and (B) the miotic response to intravitreal injection of 3 nmole of SP into both eyes after prior intravitreal injection of 30 nmole of SP antagonist into the left eye (LE) and 0.9 percent saline into the right eye (RE). The difference between the left eye and right eye following injection of SP (the 4- to 6-hour interval) was highly significant ( $P < .001$ ) with respect to both AFR and miosis. Three rabbits were tested; vertical lines give the standard error of the mean.

before irradiation of the iris. Statistical analyses were based on Student's *t*-test or regression analysis.

Intravitreal injection of SP in the two highest doses produced strong miosis and a slight AFR. The lower doses of SP produced moderate miosis and no AFR (Fig. 1). Topical application of atropine affected neither the miosis nor the AFR after injection of SP (not shown). Infra-red irradiation of the iris caused prompt miosis and a strong AFR. Topical appli-

cation of atropine before irradiation of the iris did not prevent either the miotic response or the AFR; on the contrary, the AFR was aggravated by approximately 20 percent when atropine was applied ( $P < .05$ ,  $N = 5$ ).

In itself, intravitreal injection of low doses of [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP had no effects on the eye. The two highest doses tested gave a moderate, rather long-lasting miosis (Figs. 2 and 3); the corneal sensitivity was not affected. Intravitreal

injection of 0.9 percent saline resulted in a small AFR. Injection of the SP analog produced an even smaller AFR than that seen after injection of 0.9 percent saline (Figs. 2 and 3). Prior treatment with [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP by intravitreal injection reduced the inflammatory response to SP (Fig. 2). The inflammatory response to infrared irradiation was dose dependently reduced by treatment with [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP. The highest dose tested greatly reduced the AFR (by more than 80 percent) and also inhibited miosis (Fig. 3). Combined treatment with atropine and the SP analog had the same effects as treatment with the SP analog alone (not shown). Treatment with 3 nmole of SP did not reduce the effect of subsequent irradiation of the iris (three rabbits) or of another SP injection 3 hours later (three rabbits) (not shown), a finding that seems to exclude the involvement of desensitization—that is, the development of tolerance upon repeated administration of a drug. It is interesting that [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP could be administered topically to give the same effects on the AFR and miosis as after intravitreal injection, although larger amounts had to be given (Fig. 4).

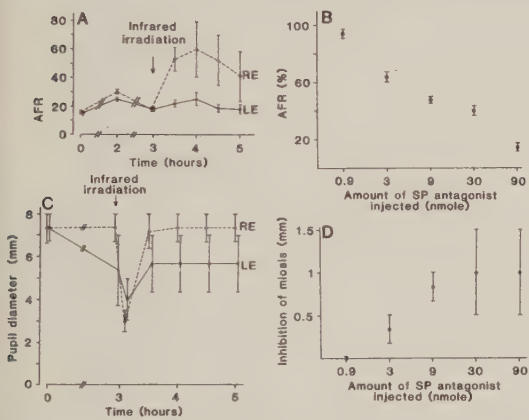


Fig. 3 (left). (A and B) The AFR and (C and D) the miotic response to infrared irradiation of the iris of both eyes after prior intravitreal injection of 90 nmole of [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP into the left eye (LE) (three rabbits) and 0.9 percent saline into the right eye (RE). The difference between the left and right eye after irradiation was highly significant ( $P < .001$ ) with respect to AFR. (B and D) Dose-response curves showing the effect of intravitreally injected SP antagonist on (B) the AFR and (D) the miosis after infrared irradiation of the iris. The AFR in the eye treated with the SP antagonist is expressed as the percentage of the AFR in the control eye at the time of maximum response. Inhibition of miosis is expressed as the difference in pupillary diameter between the eye treated with the SP antagonist and the control eye measured immediately after cessation of irradiation. The correlation coefficient (*r*) for AFR versus the amount of SP analog was .966 ( $P < .001$ ). The regression for inhibition of miosis was not significant, although the overall inhibition was clearly significant ( $P < .01$ ). There were three rabbits in each group; vertical lines give the standard error of the mean.

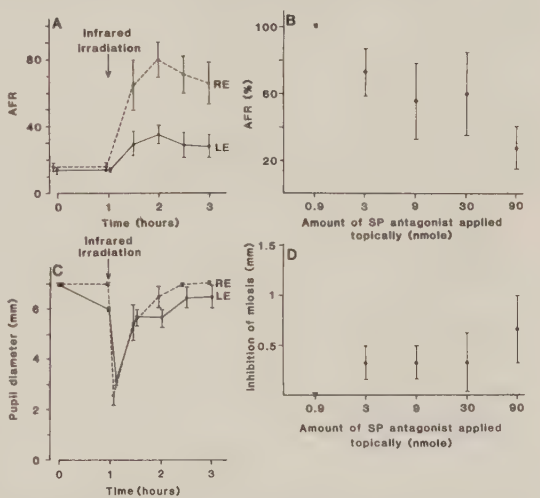


Fig. 4 (right). (A and B) The AFR and (C and D) the miotic response to infrared irradiation of the iris. The AFR in the eye treated with the SP antagonist is expressed as the percentage of the AFR in the control eye at the time of maximum response. Inhibition of miosis is expressed as the difference in pupillary diameter between the eye treated with the SP antagonist and the control eye measured immediately after cessation of irradiation. The regression for AFR versus the amount of SP analog was not significant, although the overall decrease was highly significant ( $P < .001$ ). The regression for inhibition of miosis was not significant but the overall inhibition was significant by  $P < .01$ . Three rabbits were in each group; vertical lines give the standard error of the mean.

The inflammatory response to trauma in the eye may be mediated by neurogenic factors, conceivably through axon reflexes (2, 10). Cholinergic mechanisms do not seem to be involved (2). Prostaglandins have been proposed as mediators of inflammatory responses to certain ocular trauma, such as anterior chamber paracentesis and laser irradiation of the iris (20). Conceivably also prostaglandins act via neurogenic mechanisms; an intact sensory innervation of the eye is a prerequisite for prostaglandin-evoked responses in the rabbit eye (21). Intracameral injection of SP evokes effects similar to those seen after acute trauma to the eye (5). Therefore, since SP exists in nerve fibers of the uvea (12, 13), SP may be one of the anticipated neurogenic mediators of the vasodilatation, disruption of the blood-aqueous barrier, and miosis associated with inflammation in the eye. Intravitreal application of [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP greatly reduced not only the effects of exogenous SP but also the inflammatory response to trauma to the eye. Since [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP is a specific SP antagonist (16), these observations support the view that SP is one of the neurogenic mediators of the inflammatory response [see (22)], and moreover suggest a possible clinical use of SP antagonists in alleviating inflammatory symptoms in the eye, particularly since topical application was sufficient to reduce inflammation. The corneal sensitivity seemed unaffected by treatment with the SP antagonist, suggesting that the sensory afferents in the cornea do not depend upon SP for nociception (13, 23).

G. HOLMDAHL

Department of Experimental  
Ophthalmology I,  
University of Lund, Lund, Sweden

R. HÅKANSON, S. LEANDER

Department of Pharmacology,  
University of Lund

S. ROSELL

Department of Pharmacology,  
Karolinska Institute,  
Stockholm, Sweden

K. FOLKERS

Institute for Biomedical Research,  
University of Texas, Austin 78701

F. SUNDLER

Department of Histology,  
University of Lund

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## BRADYKININ CONTRACTS THE PUPILLARY SPHINCTER AND EVOKES OCULAR INFLAMMATION THROUGH RELEASE OF NEURONAL SUBSTANCE P

G. BYNKE, R. HÅKANSON \*, J. HÖRIG and S. LEANDER

*Departments of Pharmacology and Experimental Ophthalmology I, University of Lund, Lund, Sweden and Ferring AG, Kiel, F.R.G.*

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Bradykinin contracts the isolated rabbit sphincter pupillae muscle. The contraction produced by  $10^{-8}$  M bradykinin was resistant to atropine but not to tetrodotoxin, suggesting a non-cholinergic nervous mechanism. The contraction was blocked by specific substance P (SP) antagonists, suggesting the involvement of SP. The SP antagonists tested were [D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>]SP-(1-11) and [Arg<sup>3</sup>,D-Trp<sup>7,9</sup>]SP-(5-11). The bradykinin-induced contraction exhibited marked tachyphylaxis in contrast to that induced by SP. It appears that the tachyphylaxis reflects the depletion of a bradykinin-sensitive neuronal pool of SP. Injection of bradykinin into the vitreous chamber of the rabbit eye caused miosis and disruption of the blood-aqueous barrier (manifested as aqueous flare). A second administration of bradykinin a few hours after the first injection evoked a reduced response; the response to SP upon repeated administration was unchanged. Atropine was without effect on the response to bradykinin whereas tetrodotoxin and the SP antagonists reduced the response. The results suggest that bradykinin causes miosis and aqueous flare at least partly through local release of neuronal SP.

|            |        |               |                     |                        |
|------------|--------|---------------|---------------------|------------------------|
| Bradykinin | Miosis | Aqueous flare | Substance P release | Substance P antagonist |
|------------|--------|---------------|---------------------|------------------------|

### 1. Introduction

Bradykinin, a nonapeptide found in blood, is thought to be involved in the mediation of several pathophysiological conditions, including inflammation and pain generation (Eisen, 1970; Rocha e Silva, 1970; cf. Erdös, 1979). It is widely accepted that, like kinins in general, bradykinin arises from the proteolytic degradation of plasma proteins. On the other hand, there is evidence that bradykinin is a neuronal constituent (Inouye et al., 1961; Hori, 1968; Correa et al., 1979). Not only its site of origin but also its mechanism of action is unknown. Intraocular administration of bradykinin causes miosis and breakdown of the blood-aqueous barrier with a consequent rise in the amount of protein in the aqueous humour (Cole

and Unger, 1974; Eakins, 1977; Butler and Hammond, 1980). Butler et al. (1981) and Zhang et al. (1982) recently reported that the bradykinin-induced contraction of the isolated rabbit sphincter pupillae muscle displayed tachyphylaxis and that trigeminal denervation prevented the contractile response to bradykinin but not to substance P (SP) (see also Butler and Hammond, 1980). They suggested that bradykinin acts by releasing a neurogenic mediator, possibly SP (Butler et al., 1981; Zhang et al., 1982). SP nerve fibres have previously been implicated in the non-cholinergic neuronal contraction of the sphincter pupillae muscle (Leander et al., 1981) and SP is thought to be one of the mediators of the ocular response to injury (see for instance Holmdahl et al., 1981; Zhang et al., 1982). The present report is concerned with the mechanism of action of bradykinin with respect to its effect on the isolated rabbit iris sphincter pupillae and on the rabbit eye in vivo.

\* To whom all correspondence should be addressed: Farmakologiska Inst., Sölvegatan 10, S-223 62 Lund, Sweden.

## 2. Materials and methods

### 2.1. Studies on the sphincter pupillae muscle preparation

The sphincter pupillae muscle of the rabbit iris was excised, opened and mounted vertically on a Perspex holder in a 7 ml organ bath maintained at 35°C. One end was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force displacement transducer or to a photoelectric transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Platinum ring electrodes were placed around the muscle with a constant electrode distance of 5 mm and the electrodes were connected to a Grass S 4C stimulator for field stimulation with square wave pulses (15 V over the electrodes, 0.5-1 ms duration). The muscles were stimulated with trains of pulses lasting 10 s and with a frequency of 5-20 Hz, the resting period between stimulations being at least 2 min. The contractile responses to bradykinin and SP were analyzed by using atropine, tetrodotoxin (TTX) and two SP antagonists. TTX is a well-known blocker of nervous conduction (Kao, 1966). The SP antagonists were [D-Pro<sup>2</sup>,D-Trp<sup>7-9</sup>]SP-(1-11) (Holmdahl et al., 1981; Leander et al., 1981) and [Arg<sup>5</sup>,D-Trp<sup>7-9</sup>]SP-(5-11). The pA<sub>2</sub> for [D-Pro<sup>2</sup>,D-Trp<sup>7-9</sup>]SP-(1-11) is 6.1 (Håkanson et al., 1982) and the corresponding value for [Arg<sup>5</sup>,D-Trp<sup>7-9</sup>]SP-(5-11) is 6.0 (Leander et al., unpublished observation). The mechanical activity of the preparation was recorded continuously on a Grass model 7 or model 5 polygraph. The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 133, NaHCO<sub>3</sub> 16.3, KCl 4.7, MgCl<sub>2</sub> 1.0, NaH<sub>2</sub>PO<sub>4</sub> 1.4, CaCl<sub>2</sub> 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO<sub>2</sub> in O<sub>2</sub> giving a pH of 7.2-7.3.

### 2.2. Studies on the eye

In another series of experiments we examined the effects of atropine, TTX and of the two SP antagonists on the response of the rabbit eye to intravitreal injections of bradykinin or SP. Adult pigmented rabbits (1.5-3 kg) of mixed strain were

anaesthetized with methohexital sodium (Brietal<sup>®</sup>, Lilly; 5 mg/kg) when given injections into the eye. No anaesthesia was administered during the rest of the experiments. The animals did not suffer any obvious pain or discomfort as a result of the administration of bradykinin or SP. The time course of the barrier damage induced by intravitreal injection of bradykinin was followed by photoelectric measurement (Bengtsson et al., 1975) of the aqueous flare response (AFR). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been established (Anjou and Krakau, 1961). The results are expressed in arbitrary units with reference to a standard (Anjou and Krakau, 1961). The AFR and pupillary diameter were measured every 30 min unless otherwise stated. Bradykinin (375 pmol in 15 µl if not otherwise stated), SP (30 pmol in 9 µl) or 0.9 percent saline (9 or 15 µl) was injected into the corpus vitreum by means of Hamilton precision syringes, 3-4 mm posterior to the limbus. Atropine 1% and the SP antagonists (90 nmol) were applied topically (Holmdahl et al., 1981) in a volume of 60 µl to the left eye. Atropine was administered 1 h before the injection of bradykinin. TTX (15 nmol in 10 µl) was injected intravitreally 4 h before bradykinin or SP. The SP antagonists were given twice, 1.5 h and 1 h before bradykinin. If not otherwise stated, the right eye received 0.9 percent saline topically before the injection of bradykinin.

### 2.3. Drugs

Bradykinin and SP were purchased from Beckman, Geneva, Switzerland. The SP analogues were synthesized by techniques analogous to those described elsewhere (Folkers et al., 1981; Rosell et al., 1982). Their purity was tested by high performance liquid chromatography and found to be better than 98%. They have been identified as specific SP antagonists (Leander et al., 1981; to be published) and [D-Pro<sup>2</sup>,D-Trp<sup>7-9</sup>]SP-(1-11) has previously been found not to block the motor response of the isolated taenia coli to bradykinin (Leander et al., 1981). TTX was obtained from

Sankyo, Tokyo, Japan. Atropine sulfate was from Alcon, Fort Worth, Texas, U.S.A.

### 3. Results

#### 3.1. Effect of bradykinin on the isolated sphincter pupillae muscle

The addition of SP to the isolated sphincter pupillae muscle produced a slow contraction, reaching a maximum 120-150 s after the addition to the bath (fig. 1). The bradykinin-induced contraction was even slower in onset. The concentra-

tion range studied was  $10^{-9}$ - $10^{-6}$  M. A submaximal bradykinin concentration of  $10^{-8}$  M produced a contractile response of about  $20.6 \pm 5.8\%$  (13) (mean  $\pm$  S.E.M.) (n) of the response to  $10^{-5}$  M carbachol; the corresponding value for  $10^{-8}$  M SP was  $23 \pm 3\%$  (6). Repeated administration of SP ( $10^{-8}$  M) produced unchanged responses, whereas the response to bradykinin ( $10^{-8}$  M) displayed marked tachyphylaxis (fig. 1). All preparations tested (8 separate experiments) responded in the same way: A contraction upon the first but not upon the second application of bradykinin despite extensive and repeated washings for at least 10 min between applications. Because of the

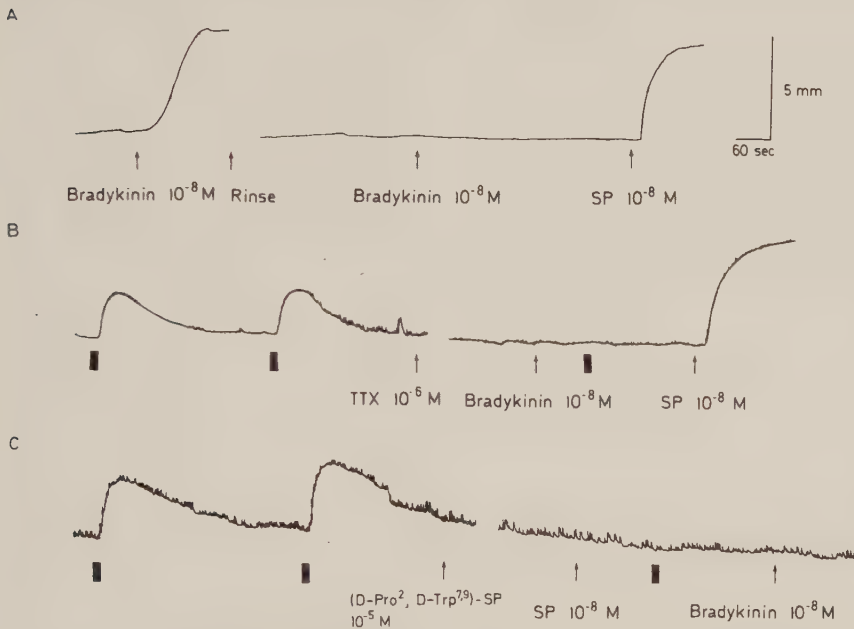


Fig. 1. Tracings of in vitro experiments with the isolated rabbit iris sphincter pupillae muscle. Atropine ( $10^{-6}$  M) was present throughout. The muscles were stimulated electrically (10 Hz, 10 s) (indicated by black rectangles). A. Contractile responses to bradykinin and SP. Note lack of effect of second application of bradykinin despite extensive washing for at least 10 min between applications. B and C. Effects of TTX (B) and the SP-blocking agent [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP-(1-11) (C) on contractile responses to electrical stimulation, bradykinin (first application), or SP. The bradykinin-induced contraction was inhibited by TTX and by the SP antagonist [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]SP-(1-11). Results with [Arg<sup>5</sup>, D-Trp<sup>7,9</sup>]SP-(5-11) were identical. The preparations were allowed to rest for about 10 min after the addition of TTX or SP to the bath. Parallel experiments without atropine showed that the contractile response to carbachol was unaffected by TTX, SP antagonists or bradykinin tachyphylaxis.

tachyphylaxis, subsequent experiments with bradykinin were performed on a first application basis. After the sphincter pupillae muscle had been made insensitive to bradykinin it retained its capacity to respond to SP (tested in 4 separate experiments). The contractile response to bradykinin, but not that to SP, was abolished by pretreatment with TTX (fig. 1). These results indi-

cate a neuronal mediation of the response to bradykinin. The contractile responses to SP and bradykinin were greatly reduced or abolished by pretreatment with either of the two SP antagonists ( $10^{-5}$  M) (fig. 1), suggesting that SP is involved in the bradykinin-evoked response.

The sphincter pupillae muscle responded to electrical stimulation with a slow contraction,

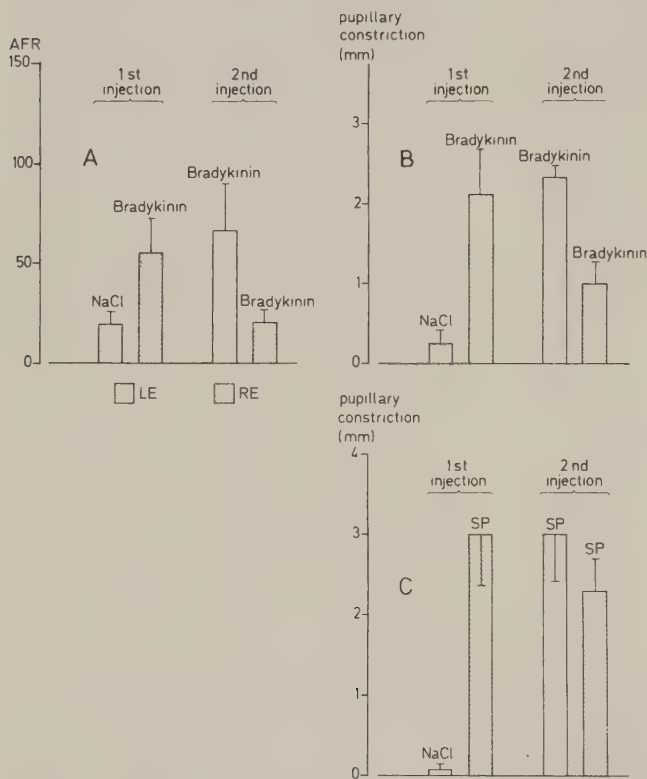


Fig. 2. The AFR and miotic response (pupillary constriction in mm) to intravitreal injections of 250 pmol ( $10 \mu\text{l}$ ) bradykinin (A, B) or 30 pmol SP (C) (right eye, RE) or 0.9 percent saline (left eye, LE) followed 3-4 (pupillary constriction) or 4-5 (AFR) h later by injection of the same dose of bradykinin or SP into both eyes. After the first injection of bradykinin the eyes became refractory to the second injection. With respect to AFR, the difference between the left and right eye was significant (Student's *t*-test,  $P < 0.05$ ) for both the first and the second injection. With respect to pupillary constriction, the *P* value was  $< 0.005$  for the first injection and  $< 0.01$  for the second injection. With SP the eyes remained responsive to the second injection. The SP dose tested was too small to have more than a marginal effect on AFR (cf Holmdahl et al., 1981) and only the miotic response was measured. Each group comprised six (SP) or eight (bradykinin) rabbits; vertical lines give the standard error of the mean.

reaching a maximum 20-30 s after cessation of stimulation (fig. 1). Atropine reduced the contractile response to low frequency (5-10 Hz) stimulation by about 25%; responses to high frequency (10-20 Hz) stimulation were not much affected (see also Tornqvist et al., 1982). The atropine-resistant contraction could be abolished by either of the two SP antagonists, indicating that the non-cholinergic contraction is mediated by SP (fig. 1) (cf. Leander et al., 1981).

### 3.2. Effect of bradykinin on the eye

Intravitreal injection of bradykinin or SP produced AFR and miosis. Repeated administration of bradykinin (250 pmol in 10  $\mu$ l) 3-5 h later evoked a much reduced response (fig. 2). In contrast, the response to repeated administration of

SP (3 h interval) was unchanged. Topical application of atropine 1 h before the injection of bradykinin did not prevent either the miotic response or the AFR, whereas intravitreal injection of TTX completely abolished the response to bradykinin (fig. 3) without affecting the response to SP. Maximal miosis was obtained 3.5 h after intravitreal injection of SP. There was no significant difference between the TTX-treated eye and the saline-injected (control) eye: 5.2 mm constriction  $\pm 0.44$  (S.E.M.) versus  $4.5 \pm 0.58$  (n = 3). In itself, topical application of the two SP antagonists had no effects on the eye (see also Holmdahl et al., 1981) but the inflammatory response to the subsequent injection of bradykinin (or SP) was greatly suppressed (fig. 3). TTX in itself caused a slight dilatation of the pupil but produced no AFR.

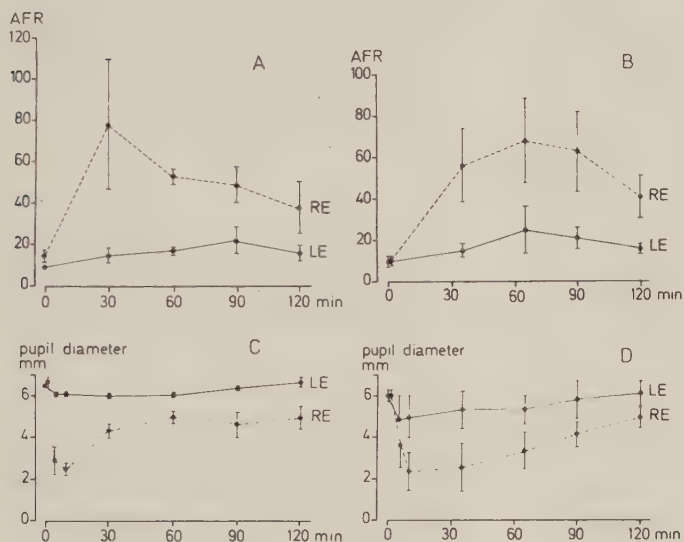


Fig. 3. The AFR (A, B) and the pupil diameter (mm) (C, D) in response to intravitreal injection of 375 pmol (15  $\mu$ l) of bradykinin into both eyes 4 h after the intravitreal injection of 15 nmol of TTX (A, C) or 1 h after topical application of 90 nmol of [Arg<sup>5</sup>,D-Trp<sup>7,9</sup>]SP-(5-11) (B, D) onto the left eye (LE) and 0.9 percent saline onto the right eye (RE). The difference between the left eye and right eye following injection of bradykinin was highly significant in both series of experiments (analysis of variance,  $P < 0.01$  in A,  $P < 0.001$  in B-D). Three rabbits were tested in each series; vertical lines give the standard error of the mean. Similar inhibition of the response to bradykinin was obtained after topical application of 300 nmol of [D-Pro<sup>2</sup>,D-Trp<sup>7,9</sup>]SP-(1-11).



#### 4. Discussion

Like SP, bradykinin is known to evoke miosis and breakdown of the blood-aqueous barrier (Cole and Unger, 1974; Eakins, 1977), thereby mimicking the response to trauma. Bradykinin and SP probably act on separate receptors (as illustrated in fig. 1). It has been shown previously (Leander et al., 1981) that the SP antagonist [D-Pro<sup>2</sup>, D-Trp<sup>7-9</sup>]SP-(1-11) does not block the bradykinin receptor in the guinea pig taenia coli. The results of the experiments on the isolated rabbit sphincter pupillae muscle confirm previous observations by Butler et al. (1981) and Zhang et al. (1982) that, unlike SP, bradykinin produces marked tachyphylaxis. The finding that the response to bradykinin was blocked by TTX suggests that bradykinin acts by releasing a neurogenic mediator, and the fact that the response to bradykinin was abolished by the two SP antagonists suggests that SP may be involved. The tachyphylaxis to bradykinin may be accounted for by exhaustion of a releasable SP pool. Desensitization of the bradykinin receptor is an unlikely explanation since extensive washing of the preparation failed to prevent the tachyphylaxis.

Intravitreal administration of bradykinin to the rabbit eye caused miosis and AFR. These responses could not be abolished by atropine (see also Cole and Unger, 1974) but they can be prevented by sensory denervation (Butler and Hammond, 1980). Not unexpectedly, the response to bradykinin was abolished by pretreatment with TTX. The effects of bradykinin on the eye thus seem to depend upon the presence of functioning sensory nerves (see also Zhang et al., 1982). Further, repeated injections of bradykinin but not SP evoked tachyphylaxis, suggesting that bradykinin acts by releasing a mediator of the inflammatory response and that this store can be exhausted by bradykinin. The observation that specific SP antagonists inhibited the response to bradykinin supports the view that SP is involved.

The response evoked by chemical and physical trauma to the eye is thought to depend upon agents released from peripheral sensory nerve fibres (Butler and Hammond, 1977; Holmdahl et al., 1981; Zhang et al., 1982). SP is a likely candi-

date for the following reasons: (1) Exogenous SP causes miosis and a slight disruption of the blood-aqueous barrier (Bill et al., 1979; Butler and Hammond, 1980; Holmdahl et al., 1981; Stjernschantz et al., 1981); (2) capsaicin, which acts by releasing SP from sensory nerve fibres (Jessell et al., 1978), evokes inflammation (Camras and Bito, 1980; Bynke, 1983); (3) SP is a constituent of sensory nerve fibers (Otsuka and Konishi, 1977), probably including those of the trigeminal nerve (Olgart et al., 1977; Bill et al., 1979; Brodin et al., 1981; Tervo et al., 1981; Tornqvist et al., 1982); (4) sectioning of the trigeminal nerve lowers the SP level in the uvea (Butler et al., 1980; Unger et al., 1981) and results in suppression of the ocular response to capsaicin, bradykinin and prostaglandins (Butler and Hammond, 1980; Butler et al., 1981; Zhang et al., 1982); (5) specific antagonists to SP prevent disruption of the blood-aqueous barrier evoked by infrared irradiation of the iris (Holmdahl et al., 1981) and by bradykinin.

The present results support the view that neuronal SP plays a key role in the ocular response to injury and emphasize the potential therapeutic usefulness of SP antagonists.

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Pupillary constriction by bradykinin and capsaicin: Mode of  
action

by

G. Bynke, C. Wahlestedt and R. Håkanson

Departments of Exp. Ophthalmology and Pharmacology,  
University of Lund, Lund, Sweden

Short title: Miotic effect of bradykinin and capsaicin

## Summary

Application of bradykinin or capsaicin to the rabbit eye evoked strong miosis. The effect could be prevented by pretreatment of the eye with tetrodotoxin or a substance P (SP) antagonist. However, the miotic response could be elicited despite TTX or the SP antagonist if the dose of capsaicin or bradykinin was increased. Bradykinin and capsaicin contracted the isolated rabbit sphincter pupillae muscle. The contraction produced by bradykinin and capsaicin was unaffected by TTX but reduced by specific SP antagonists. This indicates that bradykinin and capsaicin exert their effects on the isolated sphincter muscle through release of SP but independent of neuronal conduction. In vivo, the situation seems to be different. The finding that TTX is capable of blocking the miotic response to moderate doses of bradykinin and capsaicin suggests that the effect on the eye under these circumstances are dependent upon a normal impulse traffic.



## Introduction

Intraocular administration of bradykinin is known to evoke miosis and breakdown of the blood-aqueous barrier (Cole and Unger 1974, Bito et al 1982, Bynke et al 1983a), thereby mimicking the responses to injury. Trigeminal denervation prevents the contractile response of the isolated rabbit pupillary sphincter (Butler et al 1981, Zhang et al 1982) and the miotic response to intraocular injection of bradykinin but not to substance P (SP) (Butler and Hammond 1980). It was suggested that bradykinin acts by releasing a neurogenic mediator, possibly SP (Butler et al 1981, Zhang et al 1982). Capsaicin, an SP depleting agent (cf. Nagy 1982), evokes ocular inflammation with miosis and breakdown of the blood-aqueous barrier (Camras and Bito 1980, Bynke 1983, Bynke et al 1983b). The ocular effects of bradykinin and capsaicin could be reduced either by pretreatment with the neuronal blocker tetrodotoxin (TTX) or by the SP antagonist ( $\text{D-Pro}^2$ ,  $\text{D-Trp}^{7,9}$ )-SP (Bynke et al 1983a, b), suggesting that neuronal SP is responsible for the ocular effects of both drugs.

In the present study, we have further examined the effects of bradykinin and capsaicin on the intact eye and on the isolated rabbit sphincter pupillae muscle.

## Materials and methods

### Studies on the isolated rabbit pupillary sphincter

Adult pigmented rabbits of either sex, weighing 1.5-3 kg, were killed by a blow on the neck and exsanguinated. The eyes were enucleated within 1 min after death and opened by an incision 2-3 mm dorsally to the limbus, followed by excision of the iris from the ciliary margin. The sphincter pupillae muscle was then opened, cut in half and mounted vertically on a perspex holder in a 7 ml tissue bath maintained at 33°C. The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 133, NaHCO<sub>3</sub> 16.3, KCl 4.7, MgCl<sub>2</sub> 1.0, NaH<sub>2</sub>PO<sub>4</sub> 1.4, CaCl<sub>2</sub> 2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7% CO<sub>2</sub> in O<sub>2</sub> giving a pH of 7.2-7.3. The mechanical activity was recorded isometrically using a Grass FT03 force displacement transducer and a Grass model 7 polygraph. Before starting the experiments the sphincter muscle was allowed to equilibrate for about 90 min under a constant load of 150 mg maintained during the experiments. Electrical field stimulation with square wave pulses (14-17 V over the electrodes, 0.25 ms duration) was applied by means of a pair of platinum electrodes connected to a S 4C stimulator. The muscles were stimulated with trains of pulses lasting 10 s, the pulse frequency being 20 Hz.

At the end of each experiment the muscle was exposed to a buffer solution containing 137.7 mM KCl (but no NaCl); the resulting contraction was used as an internal standard and set as 100%.

Tetrodotoxin or (D-Arg<sup>1</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>)SP ('Spantide') was applied 10 min before application of bradykinin or capsaicin. Either of the latter two compounds was present 30 min in the bath before extensive washing and a 30 min time lapse preceding further testing of the preparation.

## Studies on the eye

The miotic response to bradykinin and capsaicin was studied after application of TTX or the SP antagonists Spantide or ( $\underline{\underline{D}}$ -Pro<sup>2</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>)-SP (AP2). Adult pigmented rabbits (1.5-3 kg) of mixed strain were anaesthetized with methohexital sodium (Brietal<sup>R</sup>, Lilly; 5 mg/kg) when given injections to the eye. Bradykinin (375 pmoles or 3.75 nmoles in 10  $\mu$ l) was injected into the corpus vitreum of both eyes by means of Hamilton precision syringes, 3-4 mm posterior to the limbus. TTX (5  $\mu$ g in 10  $\mu$ l) was injected intravitreally into the left eye 4 h before bradykinin. ( $\underline{\underline{D}}$ -Arg<sup>1</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>, Leu<sup>11</sup>)-SP was applied topically (300 nmoles in 50  $\mu$ l) to the left eye 90, 60 and 30 min before bradykinin. The right eye received 10  $\mu$ l redistilled H<sub>2</sub>O before injection of bradykinin.

Capsaicin was dissolved in a vehicle, containing ethanol, Tween 80 and saline (Bynke 1983, Bynke et al 1983b). Capsaicin solution (1.6  $\mu$ moles or 16  $\mu$ moles) were administered to both eyes by retrobulbar injection (see Bynke et al 1983b). TTX (5-10  $\mu$ g in 10-20  $\mu$ l) was injected intravitreally into the left eye 4-5 h before capsaicin. ( $\underline{\underline{D}}$ -Pro<sup>2</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>)-SP was applied topically (300 nmoles in 50  $\mu$ l) to the left eye 90, 75, 60, 45, 30 and 15 min before capsaicin. The right eye received 10-20  $\mu$ l redistilled H<sub>2</sub>O before injection of capsaicin.

The pupillary constriction induced by bradykinin or capsaicin was measured constant and uniform illumination during 15 minutes in one animal at a time.

## Results

### Effects of bradykinin and capsaicin on the isolated pupillary sphincter

Bradykinin and capsaicin evoked a slow, long-lasting contraction of the iris sphincter muscle. A marked tachyphylaxis to both compounds was noted (Fig. 1). The presence of tetrodotoxin ( $10^{-6}$  M) did not affect the contrac-

tions evoked by bradykinin or capsaicin (Figs. 2 and 3). The SP antagonist ( $\underline{\text{D}}\text{-Arg}^1$ ,  $\underline{\text{D}}\text{-Trp}^{7,9}$ ,  $\text{Leu}^{11}$ )-SP reduced the contractile response evoked by bradykinin or capsaicin (Fig. 4). Following the application of capsaicin the contractile response to bradykinin was reduced, and vice versa (Fig. 5).

The response of the sphincter muscle to electrical stimulation was in initial twitch with a high amplitude followed by a slow contraction with a lower amplitude (Fig. 6). The slow component, which remains after treatment with atropine ( $10^{-6}$  M) and guanetidine ( $5 \times 10^{-6}$  M), was reduced by  $37 \pm 7\%$  after prior application of bradykinin ( $10^{-6}$  M), and by  $54 \pm 10\%$  after prior application of capsaicin ( $10^{-4}$  M) (means  $\pm$  S.E.M.;  $n = 4$ ) (Fig. 6). After exposure of the preparation to a  $\text{Ca}^{++}$ -free medium containing EGTA ( $10^{-3}$  M) during 30 min neither bradykinin ( $10^{-6}$  M) nor capsaicin ( $10^{-4}$  M) produced any contraction (Fig. 7). A response to SP could still be elicited although greatly reduced.

#### Effect of bradykinin and capsaicin on the eye

Intravitreal injection of bradykinin produced maximal miosis within 15 min. Pretreatment with TTX (intravitreal injection) greatly reduced the miotic response to 375 pmoles bradykinin but not to 3.75 nmoles bradykinin (Fig. 8). Topical application of ( $\underline{\text{D}}\text{-Arg}^1$ ,  $\underline{\text{D}}\text{-Trp}^{7,9}$ ,  $\text{Leu}^{11}$ )-SP greatly reduced the miotic response to 375 pmoles bradykinin but not to 3.75 nmoles bradykinin. Retrobulbar injection of capsaicin produced maximal miosis within 10 min. Pretreatment with TTX or the SP antagonist greatly reduced the miotic response to 1.6  $\mu$ moles capsaicin but not to 16  $\mu$ moles capsaicin (Fig. 9).

#### Discussion

The miotic response and the breakdown of the blood-aqueous barrier induced by low doses of bradykinin and capsaicin were previously shown to be reduced

by TTX and by SP antagonists (Bynke et al 1983a, b) and hence, seem to be dependent upon SP-containing neurons. The present report partially supports this conclusion in that the miotic response to low doses of bradykinin and capsaicin was reduced both by TTX and by the SP antagonist. However, the miotic response to larger doses of bradykinin and capsaicin was unaffected both by TTX and the SP antagonist. The contractile response of the isolated pupillary sphincter to bradykinin and capsaicin was resistant to TTX but reduced by SP antagonists.

The action of bradykinin and capsaicin on the pupillary sphincter display great similarities. The compounds have been proposed to evoke SP release in vivo (Bynke et al. 1983a, b, Mandahl et al. 1984) as well as in vitro (Ueda et al. 1981, Bynke et al. 1983a). In this study we have attempted to investigate further the pupillary effects of bradykinin and capsaicin and to reveal their mode of action.

In vitro, TTX, which is a blocker of nervous impulse conduction, did not affect the contraction evoked by bradykinin or capsaicin. This excludes the involvement of nervous reflex circuits or interneurons under in vitro conditions, and speaks in favour of a direct action of the two drugs on either smooth muscle or nerve terminals. In the presence of a specific SP antagonist (see e.g. Leander et al. 1981) the contractions evoked by both bradykinin and capsaicin were greatly reduced, indicating the involvement of SP. Further, the slow component of the contraction evoked by electrical transmural stimulation, which is thought to correspond to release of neuronal SP (Leander et al. 1981, Ueda et al. 1981), was reduced after application of bradykinin or capsaicin. Finally, a marked tachyphylaxis and "cross-tachyphylaxis" was noted upon repeated application of bradykinin or capsaicin suggesting



progressive exhaustion of neuronal SP rather than receptor desensitization. The results seem to imply that both capsaicin and bradykinin act via  $\text{Ca}^{++}$ -dependent release of SP, presumably from a neuronal store.

The effect of bradykinin and capsaicin on SP-containing sensory nerve endings in the isolated rabbit iris seems to be a direct one, as evidenced by the fact that the release of SP was TTX-resistant. In the intact eye, on the other hand, both bradykinin and capsaicin seem to release SP partly via a reflex mechanism, as evidenced by the fact that TTX was able to greatly reduce the miotic response.

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## Legends to figures

Fig. 1. Representative tracings of the progressively diminishing contractile responses of the isolated rabbit iris sphincter muscle to repeated applications of bradykinin (upper panel) or capsaicin (lower panel).

Fig. 2. Concentration-response curves illustrating the effect of bradykinin on rabbit iris sphincter muscle. Drawn line indicates presence of tetrodotoxin ( $10^{-6}$  M). Each value was from single applications of bradykinin. Results are expressed as percentages of the potassium induced contraction. Means of 4-10 experiments. Vertical bars give S.E.M.

Fig. 3. Concentration-response curves illustrating the effect of capsaicin on rabbit iris sphincter muscle. Drawn line indicates presence of tetrodotoxin ( $10^{-6}$  M). Each value was from single applications of capsaicin. Results are expressed as percentages of potassium induced contraction. Means of 4-6 experiments. Vertical bars give S.E.M.

Fig. 4. Effects of the SP antagonist ( $\underline{D}$ -Arg<sup>1</sup>,  $\underline{D}$ -Trp<sup>7,9</sup>, Leu<sup>11</sup>)-SP on the contractile responses to bradykinin ( $10^{-8}$  M or  $10^{-6}$  M) and capsaicin ( $10^{-5}$  M) on the isolated iris sphincter muscle. Each value was from single applications of bradykinin or capsaicin. Results are expressed as percentages of potassium induced contractions. Means of 4-6 experiments. Vertical bars give S.E.M.

Fig. 5. Representative tracings of the contractile responses of the isolated rabbit iris sphincter muscle to successive application of capsaicin ( $10^{-4}$  M), bradykinin ( $10^{-6}$  M) and SP ( $10^{-8}$  M) (upper panel), and bradykinin ( $10^{-6}$  M), capsaicin ( $10^{-4}$  M) and SP ( $10^{-8}$  M) (lower panel). The reduction of the contractile response to bradykinin after application of capsaicin, and vice versa, is referred to as "cross-tachyphylaxis" in the text. The response to SP was unaffected by either of the pretreatments.

Fig. 6. Representative tracings illustrating the effect of electrical stimulation on the isolated rabbit iris sphincter muscle before and after atropine ( $10^{-6}$  M) + guanethidine ( $5 \times 10^{-6}$  M), followed by application of bradykinin (upper panel) or capsaicin (lower panel). The subsequent contractile response to electrical stimulation is reduced in both series of experiments.

Fig. 7. The contractile effect of bradykinin (upper panel) and capsaicin (lower panel) is dependent upon  $\text{Ca}^{++}$  as illustrated in these tracings showing also the contractile effect of SP in normal and  $\text{Ca}^{++}$ -free medium. In contrast to bradykinin and capsaicin SP still evokes a contraction, albeit greatly reduced.

Fig. 8. Maximal pupillary constriction (Mean  $\pm$  S.E.M.) 5-15 minutes after intravitreal injection of 375 pmoles or 3.75 nmoles bradykinin to both eyes after pretreatment of the left eye with either TTX or Spantide. In the TTX treated groups the difference between the eyes was highly significant with 375 pmoles bradykinin,  $p < 0.005$  (Student's t-test) but not with 3.75 nmoles. In the SP antagonist treated groups the difference between the eyes was highly significant with 375 pmoles bradykinin,  $p < 0.01$  (Student's t-test) but not with 3.75 nmoles.

Fig. 9. Maximal pupillary constriction (Mean  $\pm$  S.E.M.) 5-10 minutes after retrobulbar injection of 1.6  $\mu$ moles or 16  $\mu$ moles capsaicin to both eyes after pretreatment of the left eye with either TTX or AP2. In the TTX treated groups the difference between the eyes was significant with 1.6  $\mu$ moles,  $p < 0.025$  (Student's t-test) but not with 16  $\mu$ moles. In the SP antagonist treated groups the difference between the eyes was highly significant with 1.6  $\mu$ moles,  $p < 0.005$  (Student's t-test) but not with 16  $\mu$ moles.



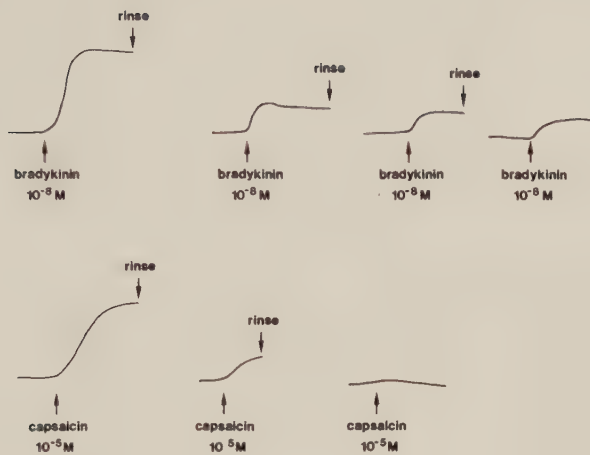


Fig.1

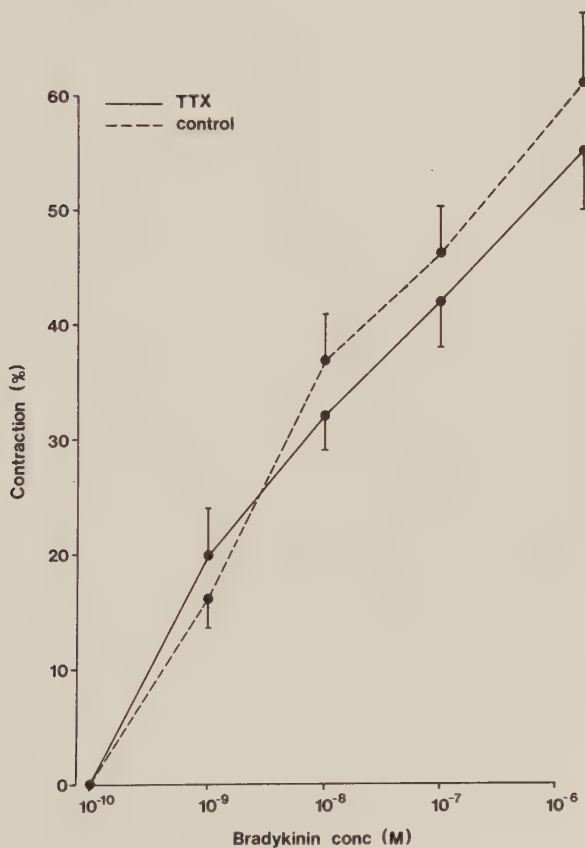


Fig.2

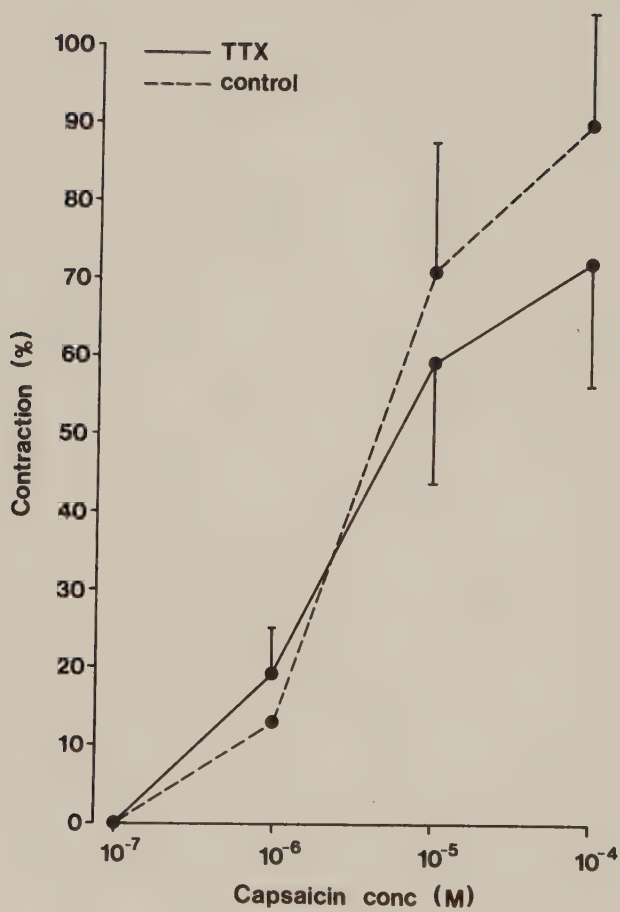


Fig.3

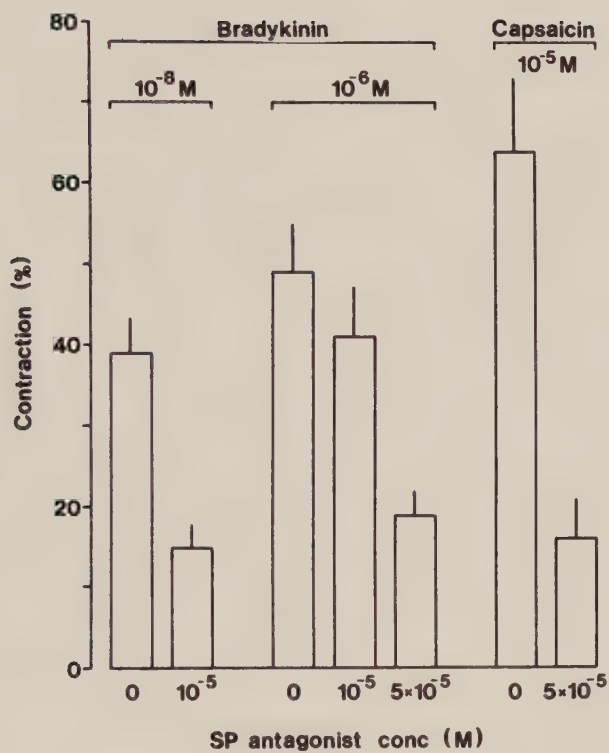
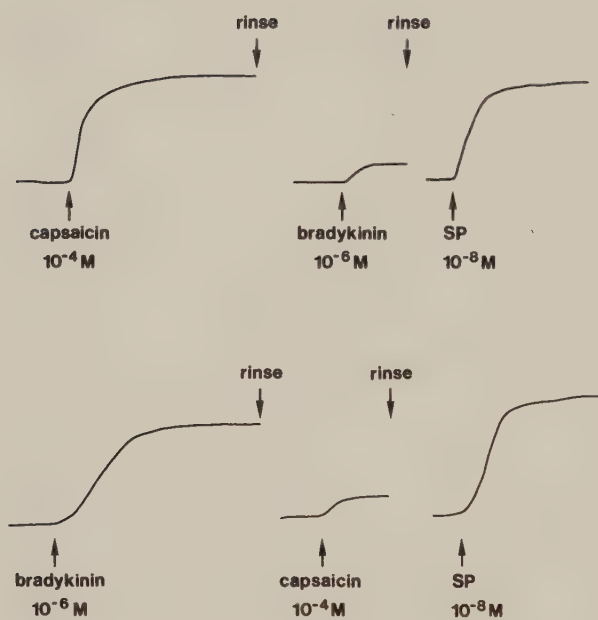
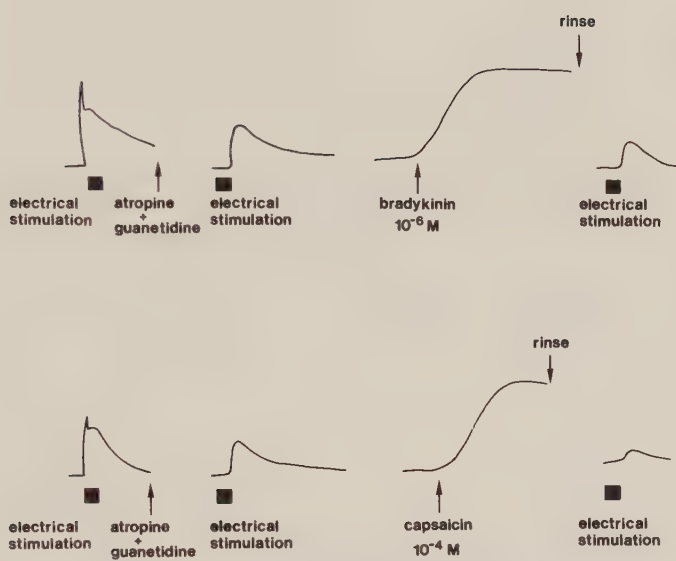


Fig.4



**Fig.5**



**Fig.6**



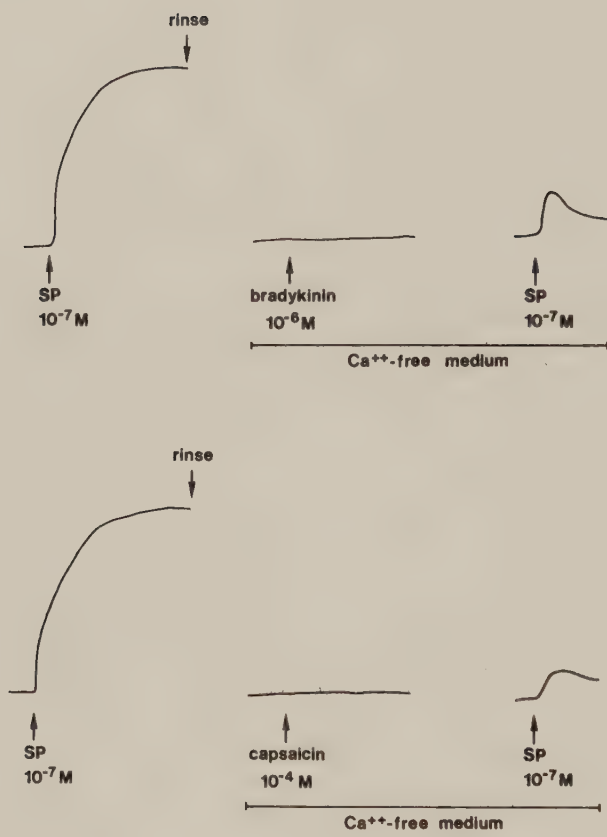


Fig.7

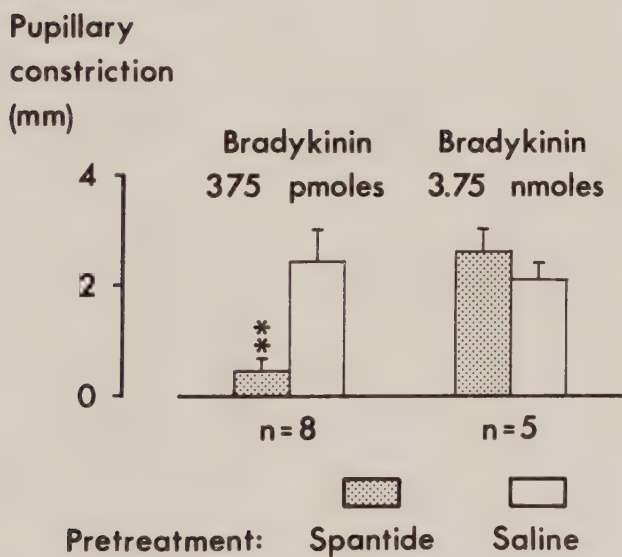
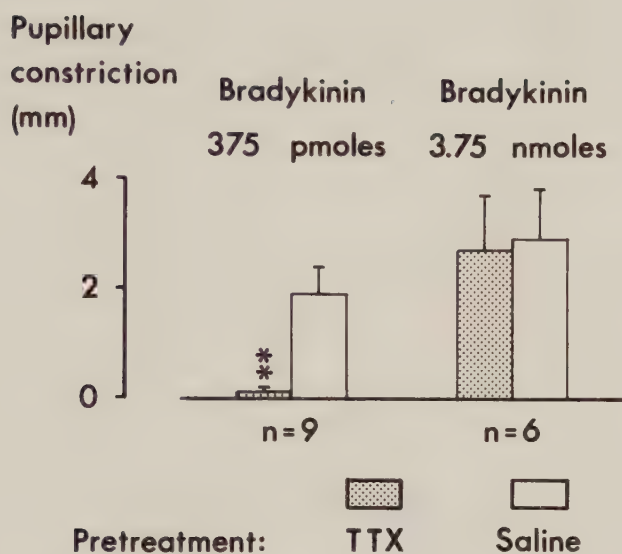


Fig.8

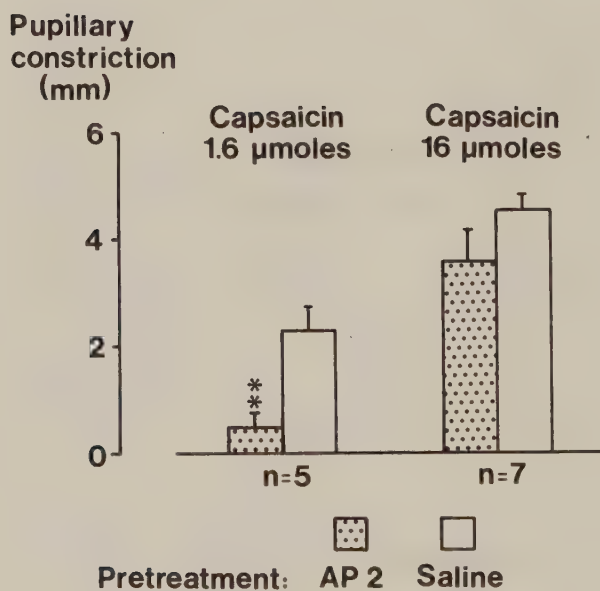
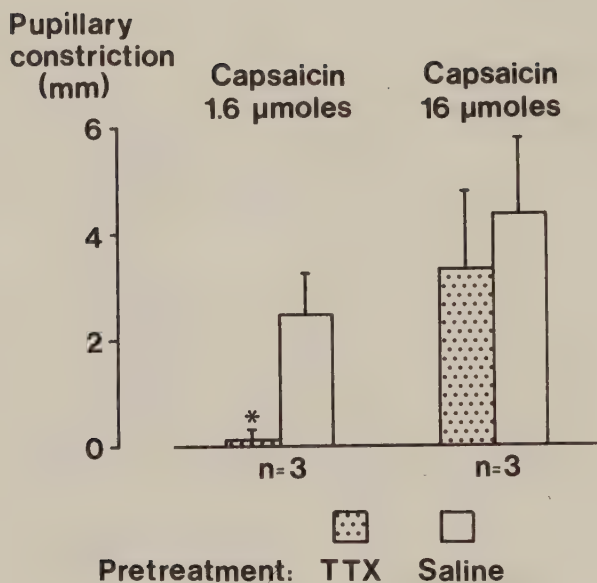


Fig.9

## Ocular responses evoked by capsaicin and prostaglandin E<sub>2</sub> are inhibited by a substance P antagonist<sup>1</sup>

G. Bynke, R. Håkanson<sup>2</sup> and J. Hörig

*Departments of Experimental Ophthalmology and Pharmacology, University of Lund, S-22362 Lund (Sweden), and Ferring Arzneimittel GmbH, D-2300 Kiel (Federal Republic of Germany), January 31, 1983*

**Summary.** Application of capsaicin or prostaglandin E<sub>2</sub> to the rabbit eye resulted in miosis and breakdown of the blood-aqueous barrier, manifested in aqueous flare. Pretreatment with the neuronal blocker tetrodotoxin or the substance P antagonist (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP<sub>1-11</sub> greatly reduced the ocular responses to capsaicin and prostaglandin E<sub>2</sub>. The results suggest a role for neuronal substance P in the ocular response to injury.

The response to ocular injury in the rabbit eye is characterized by miosis, vasodilatation and a breakdown of the blood-aqueous barrier. Among stimuli known to elicit one or more of these responses are chemical irritants, such as nitrogen mustard<sup>3</sup> and capsaicin<sup>3,4</sup>, endogenous chemicals, such as bradykinin<sup>5</sup>, prostaglandins<sup>6</sup> and substance P (SP)<sup>7-9</sup> and ocular trauma, induced, for instance, by IR-irradiation of the iris<sup>10</sup>. Following sensory denervation of the eye by diathermic destruction of the trigeminal nerve the miotic and hypertensive response to laser irradiation<sup>11</sup>, capsaicin, bradykinin and prostaglandins is prevented<sup>7,12,13</sup> whereas that to SP remains<sup>7,13</sup>. It has therefore been proposed that all chemical irritants, except SP, act to release a mediator from sensory nerve endings<sup>13</sup>. It has also been suggested that the mediator is related to SP<sup>13</sup>. Recently, it was shown that the ocular response to IR-irradiation and to bradykinin can be prevented by pretreatment of the eye with a newly developed SP antagonist<sup>14,15</sup>. The present study is concerned with the mechanism behind the ocular injury evoked by capsaicin and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

**Methods.** Adult pigmented rabbits (1.5–3 kg) of mixed strain were anesthetized with methohexital sodium (Brietal®, Lilly; 5 mg/kg) when given intravitreal or retrobulbar injections. No anesthesia was administered during the rest of the experiments. The time course of the barrier damage was followed by photoelectric measurement<sup>16</sup> of the aqueous flare response (AFR). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been

established<sup>17</sup>. The method has the advantage of being atraumatic, permitting continuous recording of the AFR during the experiment. Furthermore, the method detects changes in AFR that cannot be observed by conventional focal illumination. The results are expressed in arbitrary units with reference to a standard<sup>17</sup>. Unless otherwise indicated, the AFR and pupillary diameter were measured every 30 min. A potent blocker of nervous conduction, tetrodotoxin (TTX)<sup>18</sup>, (10 µg in 20 µl bidest H<sub>2</sub>O) was injected into the vitreous chamber of the left eye by means of a Hamilton precision syringe, 3–4 mm posterior to the limbus. The control (right) eye received 20 µl H<sub>2</sub>O. These injections were made 4 h before the administration of either capsaicin or PGE<sub>2</sub> to both eyes. The SP antagonist (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP<sub>1-11</sub> was applied topically in a volume of 50 µl to the left eye. The SP antagonist was given twice, 1 h and 30 min, respectively, before capsaicin or PGE<sub>2</sub>. The right eye (control eye) received instead 0.9% saline topically. Capsaicin (150 µl of a 0.33% solution) was administered to both eyes by retrobulbar injection using a thin needle (0.4 mm diameter, 20 mm length), introduced through the center of the lower eyelid, near the lid margin, and around the interior aspect of the globe to a depth of 10 mm. Aspiration was invariably performed to make sure that the injection was not into the venous sinus. PGE<sub>2</sub> (0.85 µg in 5 µl) was applied topically onto both eyes. The doses of capsaicin and PGE<sub>2</sub> were selected because they produced small but consistent effects in the eye.

Capsaicin and PGE<sub>2</sub> were purchased from Sigma, St. Louis, Mo, USA. 50 mg of capsaicin was dissolved in 75 µl 99.5%

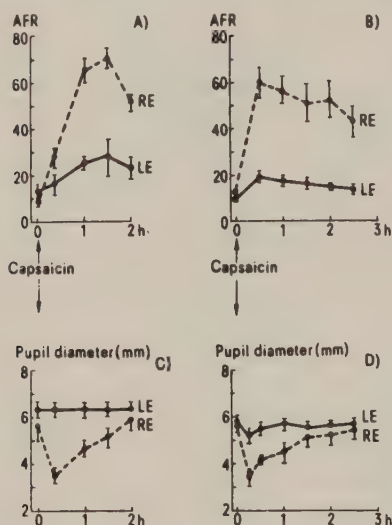


Figure 1. The AFR (A, B) and the pupil diameter (C, D) in response to retrobulbar injection of capsaisin to both eyes 4 h after the intravitreal injection of 30 nmol (10  $\mu$ g) of TTX (A, C) or 1 h after the 1st topical application of 300 nmol (470  $\mu$ g) of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP<sub>1-11</sub> (B, D) onto the left eye (LE) and 0.9% saline onto the right eye (RE). Means  $\pm$  SEM (vertical bars). Five rabbits received the SP antagonist and 3 rabbits received TTX. The difference between the left eye and right eye following injection of capsaisin was significant in both series of experiments (analysis of variance,  $p < 0.001$  in A-D).

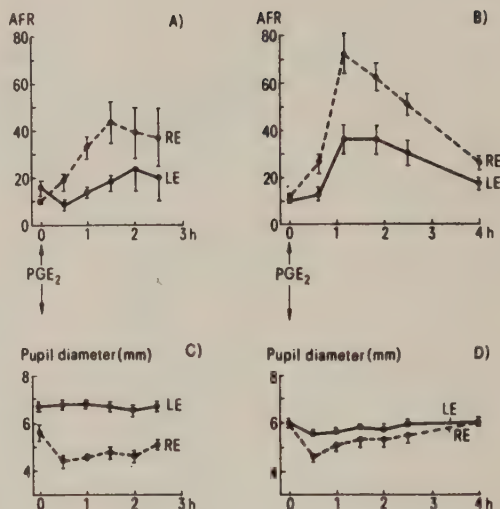


Figure 2. The AFR (A, B) and the pupil diameter (C, D) in response to topical application of PGE<sub>2</sub> onto both eyes 4 h after the intravitreal injection of TTX (A, C) or 1 h after the 1st topical application of 300 nmol (470  $\mu$ g) of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP<sub>1-11</sub> (B, D) onto the left eye (LE) and 0.9% saline onto the right eye (RE). Six rabbits received TTX and 8 rabbits received SP antagonist. Means  $\pm$  SEM (vertical bars). The difference between the left eye and right eye following application of PGE<sub>2</sub> was significant in both series of experiments (analysis of variance,  $p < 0.001$  in A-D).

ethanol, 425  $\mu$ l Tween 80 and 14.5 ml 0.9% saline to give a concentration of 0.33%. 1 mg of PGE<sub>2</sub> was dissolved in 0.1 ml ethanol and 0.9% saline was added to give a concentration of 0.17 mg/ml. The SP analogue was synthesized by techniques analogous to those described elsewhere<sup>19,20</sup>. Its purity was tested by HPLC and found to be better than 98%. It has previously been identified as a competitive antagonist of SP<sup>11,22</sup> with a selective action in that it antagonizes the smooth muscle contraction induced by SP and related tachykinins but not that induced by histamine, carbachol, 5-hydroxytryptamine, bradykinin, vasopressin, bombesin or prostaglandins<sup>21</sup>. 132 mg was dissolved in 14 ml 0.9% saline to give a concentration of 9.4 mg/ml. TTX was obtained from Sankyo, Tokyo, Japan, and dissolved in 0.9% saline.

Analysis of variance (2-way) was used for calculating the significance of difference between treated and control eyes. **Results and discussion.** Injection of capsaisin produced AFR and miosis (fig. 1). Also PGE<sub>2</sub> evoked AFR and slight miosis (fig. 2). Pretreatment with either TTX or the SP antagonist greatly reduced the responses to both capsaisin and PGE<sub>2</sub> (figs 1 and 2).

The responses to ocular injury are thought to depend at least partly upon agents released from peripheral sensory nerve fibers<sup>7,12-15</sup>. Responses to intracameral administration of capsaisin, bradykinin and prostaglandin E<sub>1</sub> were greatly reduced or abolished after sensory denervation of the eye by diathermic destruction of the trigeminal nerve<sup>7</sup>. The response to SP was not abolished<sup>7</sup>. Hence, the response to SP does not depend upon the functional integrity of the sensory nerves, and it may be that SP bears some resemblance to the endogenous antidromic mediator<sup>13</sup>. In fact, SP

has been put forward as a candidate mediator for the neurogenic response since: 1. Exogenous SP causes ocular hypertension and miosis<sup>7,8,13,23</sup>; in addition, disruption of the blood-aqueous barrier has been noted<sup>23</sup>. 2. SP is a constituent of sensory nerve fibers<sup>24</sup>, including those of the trigeminal nerve<sup>23,25-27</sup>. 3. Sectioning of the trigeminal nerve lowers the SP levels in the uvea<sup>28,29</sup> and suppresses the responses to capsaisin, bradykinin and prostaglandins, but not to SP<sup>7</sup>. 4. Capsaisin, known to release SP<sup>30</sup>, evokes signs of ocular inflammation<sup>3,31</sup>. 5. Capsaisin pretreatment prevents the ocular response to infrared irradiation and prostaglandins<sup>31</sup>. 6. Antagonists to SP suppress ocular responses to IR-irradiation, bradykinin and SP<sup>14,15</sup>. The results of the present study suggest that capsaisin and PGE<sub>2</sub> cause ocular irritation at least partly by a neurogenic mechanism, since the responses could be reduced by pretreatment with TTX. Conceivably, neuronal SP or a related peptide is involved as a mediator in the ocular response to both capsaisin and PGE<sub>2</sub> since a competitive SP antagonist greatly reduced the effects of these drugs.

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Short title: Ocular effects of SP antagonist

Long-term administration of a substance P antagonist, ( $\underline{\underline{D}}$ -Pro<sup>2</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>)-SP,  
abolishes the response to ocular trauma

G. Bynke, R. Håkanson, J. Hörig and K. Folkers

Departments of Exp. Ophthalmology I and Pharmacology, University of Lund,  
Lund, Sweden, Ferring Arzneimittel GmbH, Kiel, FRG and Institute for Bio-  
medical Research, University of Texas, Austin 78701, Texas, USA

## SUMMARY

The effects of long-term ocular administration of a substance P antagonist, (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP, was studied in the rabbit. A mild trauma was applied in the form of infrared irradiation of the iris two to 3 months after topical administration of the peptide twice daily. The aqueous flare response to the ocular trauma was abolished during ongoing treatment. Two days after interruption of treatment ocular trauma still evoked a reduced response. Another two days later ocular trauma evoked a normal response. Thus, the effect was reversible. No adverse reactions to the SP antagonist were observed.

## INTRODUCTION

A substance P antagonist was recently shown to inhibit the ocular response to infrared irradiation of the iris<sup>1</sup>. Substance P (SP) has been proposed to be a neurogenic mediator of the ocular response to trauma, since it evokes symptoms of ocular injury<sup>1-3</sup> and since it is released into the aqueous humor of the rabbit eye after stimulation of the trigeminal nerve<sup>2</sup>. The present study deals with the effects of long-term administration of an SP antagonist, ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP, onto the eye.

## MATERIALS AND METHODS

### Drug

The SP analogue was synthesized by techniques analogous to those described elsewhere<sup>4,5</sup>. Its purity was tested by high performance liquid chromatography and found to be better than 98%. It has previously been described as a competitive and fairly specific <sup>SP</sup>antagonist<sup>6,7</sup>. It was dissolved in sterile 0.9% saline. Solutions were prepared fresh every week and stored in a refrigerator.

### Single dose treatment

Six pigmented rabbits received a single dose of 300nmoles (50  $\mu\text{l}$ ) of ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP topically onto the left eye. The right eye served as control and received a single dose of 0.9% saline topically.

### Multiple dose treatment

Three pigmented rabbits received ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP topically for 2 months onto the left eye in a dose of 300 nmoles (50  $\mu\text{l}$ ) twice daily, at 8-10 a.m. and 3-7 p.m., respectively. Another group of eight pigmented rabbits received ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP, topically for 3 months onto the left eye twice daily, at 7-9 a.m. and 3-7 p.m., respectively. Three of the latter rabbits received 300 nmoles (50  $\mu\text{l}$ ) and five 30 nmoles (50  $\mu\text{l}$ ). The right eye (control) received 0.9% saline twice a day.

### Disruption of the blood-aqueous barrier

The blood-aqueous barrier was disrupted by infrared irradiation (IR)<sup>8</sup> of the iris of both eyes for 2 minutes. IR is not painful and the animals were conscious but gently restrained. The course of the barrier damage was follo-



wed by photo-electric measurements<sup>9</sup> of the aqueous flare response (AFR) every 30 minutes. This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been established<sup>10</sup>. The method has the advantage of being atraumatic, permitting recording of the AFR at intervals during the experiment. Furthermore, the method detects changes in AFR that cannot be observed by conventional focal illumination. The results are expressed in arbitrary units with reference to a standard<sup>10</sup>.

## RESULTS

Single dose treatment. Three rabbits received a single dose of 300 nmoles SP antagonist topically onto the left eye one hour before IR of the iris of both eyes. The AFR was greatly reduced in the left eye (Fig. 1 A). Another group of 3 rabbits received a single dose of 300 nmoles topically onto the left eye three hours before IR of the iris of both eyes. There was still an inhibition of the AFR in the left eye (Fig. 1 B). On another occasion the same 3 rabbits received a single dose of 300 nmoles five hours before IR of the iris of both eyes. The AFR was no longer inhibited (not shown in fig.).

Multiple dose treatment. At the 2-3 month stage and during ongoing treatment with the SP antagonist the following experiments were performed. Three animals, which had received the antagonist (300 nmoles) for 2 months, were subjected to IR of the iris of both eyes one hour after administration of the antagonist. The AFR was abolished in the pre-treated eye (Fig. 2 A). Six days later, when the antagonist had been withdrawn for two days, the AFR to IR of the iris was still reduced in the pre-treated eye (Fig. 2 B). Another two and fourteen days later, respectively, the three animals were again subjected to IR of the iris. There was no longer any inhibition of the AFR (Fig. 2 C, D).

Another group of 3 rabbits, pretreated with 300 nmoles SP antagonist for 3 months, was subjected to IR of the iris of both eyes about half an hour after pretreatment with the SP antagonist. The AFR was greatly inhibited (Fig. 3 A).

IR of the iris was also performed on the 5 rabbits treated with 30 nmoles of the SP antagonist for 3 months. The AFR was greatly reduced (Fig. 3 B).

During and after long-term treatment with the SP antagonist no adverse reactions were observed.

## DISCUSSION

The responses to ocular injury are thought to depend at least partially upon agents released from peripheral sensory nerve fibers<sup>1,11-13</sup>. SP has been put forward as a candidate mediator for the neurogenic response since exogenous SP evokes symptoms of ocular injury<sup>1-3,12</sup>, since SP occurs in trigeminal nerve fibers<sup>2,14-17</sup>, and since specific antagonists to SP suppress the responses not only to SP<sup>1</sup> but also to IR<sup>1</sup>, bradykinin<sup>11</sup>, prostaglandin<sup>18</sup> and capsaicin<sup>18</sup>.

The present work confirms and extends the previous finding that topical application of the SP antagonist inhibits the response to IR of the iris<sup>1</sup>. The inhibitory effect of a single topical administration of the antagonist persisted for 3 hours. Long-term treatment with the SP antagonist abolished the response to IR of the iris. The response to ocular trauma was reduced two days after interruption of treatment, suggesting that with time the antagonist accumulates in the eye. Another two days later IR again produced AFR. Thus, the effect was reversible. Furthermore, long-term treatment with the SP antagonist did not reveal any adverse reactions during or after 3 months of treatment with very large doses.

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## LEGENDS

Fig. 1. Aqueous flare response (AFR) (mean  $\pm$  S.E.) to infrared irradiation (IR) of the iris in the left eye (LE) and the right eye (RE). A. The left eye of 3 rabbits had been pretreated with a single dose of 300 nmoles SP antagonist one hour before IR of the iris. The difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way). B. The left eye of 3 rabbits had been pretreated with a single dose of 300 nmoles SP antagonist three hours before IR of the iris. The difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way).

Fig. 2. Aqueous flare response (AFR) (mean  $\pm$  SE) to infrared irradiation (IR) of the iris in the left eye (LE) and the right eye (RE). The left eye of 3 rabbits had been treated with 300 nmoles SP antagonist applied topically twice daily for 2 months. A. During ongoing treatment 2 months after the start; the difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way). B. Two days after interruption of treatment; the difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way). C. Four days after interruption of treatment; no significant difference between the eyes ( $p > 0.05$ , analysis of variance, two-way). D. Sixteen days after interruption of treatment; no significant difference between the eyes ( $p > 0.05$ , analysis of variance, two-way).

Fig. 3. Aqueous flare response (AFR) (mean  $\pm$  SE) to infrared irradiation (IR) of the iris in the left eye (LE) and the right eye (RE). A. The left eye of 3 rabbits had been pretreated with 300 nmoles SP antagonist applied topically daily for 3 months. The right eye had been pretreated with saline. The difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way). B. The left eye had been pretreated with 30 nmoles SP antagonist for 3 months and the right eye pretreated with saline ( $n=5$ ). The difference between the eyes is highly significant ( $p < 0.001$ , analysis of variance, two-way).



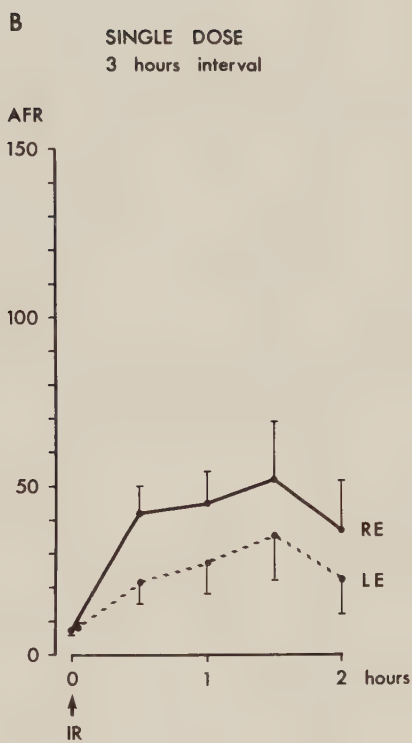
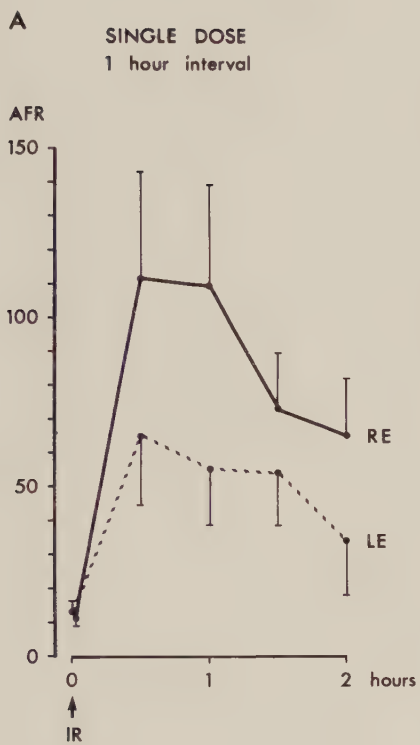


Fig.1

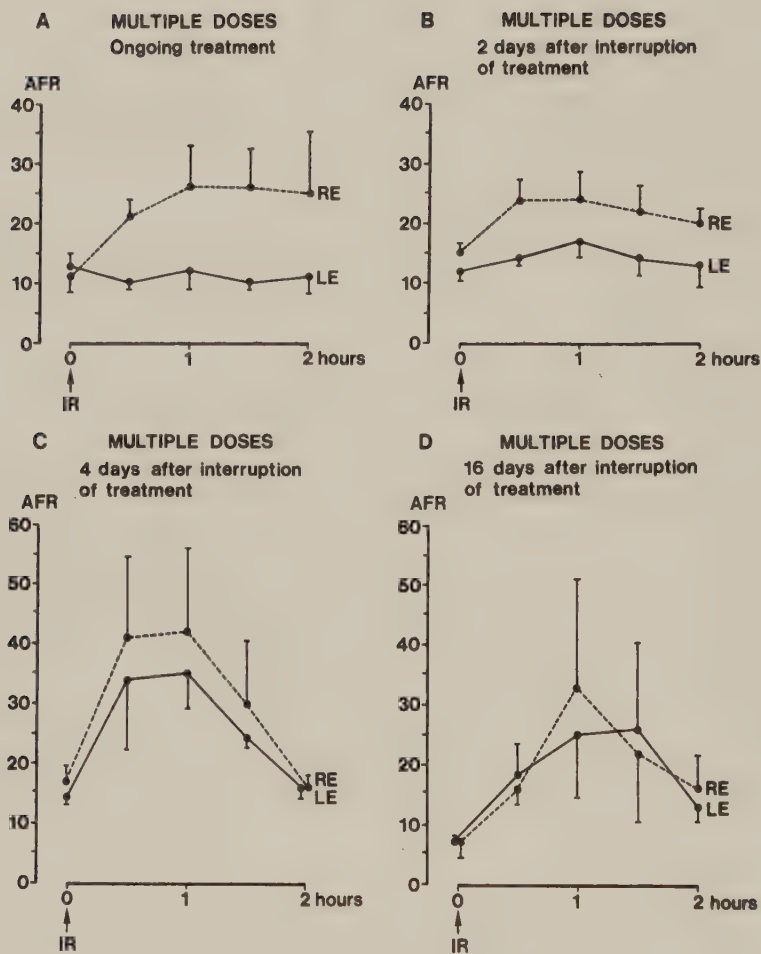
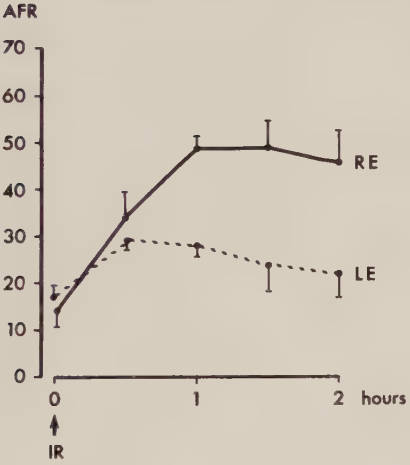


Fig.2

A

MULTIPLE DOSES  
Ongoing treatment



B

MULTIPLE DOSES  
Ongoing treatment

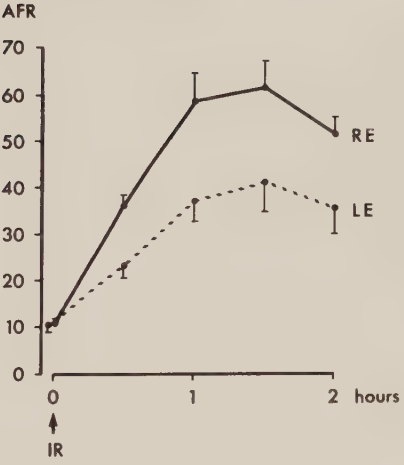


Fig.3



Short title: Substance P and corneal nociception

Is corneal substance P necessary for corneal nociception?

Gunnel Bynke, Rolf Håkanson and Frank Sundler

Departments of Exp. Ophthalmology, Pharmacology and Histology,  
University of Lund, Sweden

Correspondence to:

Dr. Rolf Håkanson

Department of Pharmacology

University of Lund

Sölvegatan 10

S-223 62 LUND/Sweden



### Abstract

G. BYNKE, R. HÅKANSON and F. SUNDLER, Is corneal substance P necessary for corneal nociception?, European J. Pharmacol. 00 (1984) 000-000.

Substance P (SP)-containing nerve fibers are few in the rabbit cornea, which is richly supplied with acetylcholinesterase-positive nerve fibers, presumably sensory in nature. Treatment with capsaicin given by retro-bulbar injection 48 h prior to sacrifice, caused SP to disappear from the SP nerves in the cornea without affecting the acetylcholinesterase-positive nerve fibers.

The corneal sensitivity to a tactile stimulus (wetted cotton swabs) and to a chemical stimulus (local application of capsaicin) and the disruption of the blood-aqueous barrier after local injury (infrared irradiation of the iris) were tested after pretreatment with capsaicin, the SP antagonist D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP (single topical application or long-term application twice daily for 2 months), the local anaesthetic oxibuprocaine (topical application), or the neuronal blocker tetrodotoxin (intravitreal injection). All these treatments abolished or reduced the disruption of the blood-aqueous barrier after local injury, but only oxibuprocaine and tetrodotoxin abolished the corneal sensitivity to tactile and chemical stimuli.

It is suggested that 1) SP nerve fibers constitute a minor proportion of the sensory nerve supply to the cornea, 2) that the neurogenic mechanisms involved in the response to ocular injury may differ from those involved in corneal nociception, and 3) that uveal SP is involved in the response to ocular trauma but that corneal SP is probably not necessary for corneal nociception.

Key-words: Substance P, substance P antagonist, capsaicin, aqueous flare, nociception, blood-aqueous barrier.

## 1. Introduction

Substance P (SP) is thought to be neurotransmitter in a population of primary sensory afferents (Lembeck, 1953; Lembeck and Gamse, 1982). In skin, mucous membranes, dental pulp, anterior segment of the eye and in several other organs SP has been localized to thin, probably unmyelinated nerve fibers, which may represent peripheral branches of sensory neurones (cf. Hökfelt et al., 1975; Olgart et al., 1977b; Tervo et al., 1981; Stone et al., 1982; Tornqvist et al., 1982). SP fibers in the eye are thought to originate in the trigeminal ganglion (Tervo et al., 1981; Keen et al., 1982). This view is supported by the finding that capsaicin, which first stimulates certain sensory nerve endings and then makes them inexcitable (Jancsó et al., 1967; Jancsó, 1968; Bynke, 1983), depletes SP from the central projections of sensory neurones (Yaksh et al., 1979; Gamse et al., 1981) and from peripheral fibers (Hayes and Tyers, 1980). There is also experimental evidence that SP may be involved in the effects caused by antidromic activation of sensory afferents (Lembeck and Holzer, 1979). The dental pulp of cats is richly supplied with SP nerve fibers originating from the trigeminal system (Olgart et al., 1977b). Electrical stimulation of the appropriate nerve brings about a release of SP in the pulp (Olgart et al., 1977a) simultaneously with an atropine-resistant vasodilatation (Gazelius and Olgart, 1980). Intra-arterial administration of an SP antagonist, D-Pro<sup>2</sup>, D-Phe<sup>7</sup>, D-Trp<sup>9</sup>-SP, blocks this vasodilatation (Rosell et al., 1981). Similarly, in the eye the inflammatory response to injury is known to be mediated at least in part by local axon reflexes in which afferent nociceptive neurones are involved (Unger et al., 1977; Butler et al., 1980). The inflammatory response to ocular injury includes miosis, increased intraocular pressure and breakdown of the blood-aqueous barrier. SP has been shown to be released into the aqueous humor of the rabbit eye after stimulation of the trigeminal nerve (Bill et al., 1979). Injection of SP into the anterior chamber of the rabbit eye evokes responses similar to those seen after acute injury (Bill et al., 1979). Further evidence that SP may be a neurogenic mediator of inflammatory responses is provided by the finding that administration of SP antagonists to the rabbit eye reduces the breakdown of the blood-aqueous barrier in response to the injury caused by infrared irradiation of the iris (Holmdahl et al., 1981) and to bradykinin (Bynke et al., 1983a), prostaglandin E<sub>2</sub> or capsaicin (Bynke et al., 1983b).

Additional evidence that SP may function as a transmitter in afferent nerve fibers involved in nociception comes from the observation that intraspinal injection of SP in mice shortens the tail withdrawal time upon exposure to heat (Lembeck et al., 1981). Interestingly, SP antagonists administered intraspinally have antinociceptive effects and also block the hyperalgesic effect of SP (Lembeck et al., 1981).

The cornea is rich in sensory nerve fibers thought to derive from the trigeminal nerve (Zander and Weddel, 1951). It has been suggested that acetylcholinesterase staining reveals sensory nerve fibers in the cornea (Tervo, 1976). Despite its rich sensory innervation there is a paucity of SP fibers (Tervo et al., 1981; Stone et al., 1982; Ehinger et al., 1983). The question arose, therefore, is corneal SP involved in corneal nociception?

## 2. Materials and Methods

Adult pigmented rabbits (1.5–3 kg) of mixed strain were anaesthetized with methohexital sodium (5 mg/kg) when given intravitreal or retrobulbar injections. No anaesthesia was administered during the rest of the experiments. A 1% capsaicin solution was prepared by dissolving 100 mg of capsaicin in 150  $\mu$ l of 99.5% ethanol; then 850  $\mu$ l of Tween 80 was added, followed by 9 ml of saline. 0.5 ml of 1% capsaicin was administered by retrobulbar injection to the left eye of 5 rabbits. This causes disruption of the blood-aqueous barrier. Topical application of capsaicin is without effect on the blood-aqueous barrier. Another group of 3 rabbits received tetrodotoxin (TTX) (Kao, 1966) (10  $\mu$ g in 20  $\mu$ l) into the left eye by intravitreal injection, 3 to 4 mm posterior to the limbus. TTX does not penetrate the cornea and cannot be given topically (Unger et al. 1977). TTX was dissolved in redistilled  $H_2O$ . Another group of 5 rabbits was treated with the specific SP antagonist, D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP (Leander et al., 1981; Håkanson et al., 1982), administered in a dose of 300 nmoles topically onto the left eye twice daily for two months. The SP antagonist was dissolved in sterile 0.9% saline. Solutions were prepared fresh every week and stored in a refrigerator. Another group of 3 rabbits received 50  $\mu$ l of 0.4% oxibuprocaine, a local anaesthetic drug, topically onto the left eye. A control group of 5 rabbits received no treatment at all.

The corneal sensitivity to a tactile stimulus was tested by the use of wetted cotton swabs. The sensitivity to a chemical stimulus was tested by instillation of a drop of a 0.12% capsaicin solution (0.9% saline) onto the eye and counting the ensuing number of wipings with the forepaws (Szolcsányi and Jancsó-Gabor, 1975).

Disruption of the blood-aqueous barrier with subsequent leakage of protein into the anterior chamber was elicited by infrared irradiation (IR) of the iris for two minutes. The time course of the barrier damage was followed by photo-electric measurement of the aqueous flare response (AFR) (Bengtsson et al., 1975). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the blood-aqueous barrier. A correlation between the AFR and the protein concentration has been established (Anjou and Krakau, 1961).

## 2.2 Histochemistry

The cornea was dissected out from the eyes of rabbits immediately after death (intravenous injection of air). Whole mounts were prepared by stretching the cornea onto chrome alun-coated glass slides which were then allowed to dry for a few minutes. Some of the whole mounts were fixed in Stefanini's fixative for 18 hours, rinsed in 10% sucrose, dehydrated and rehydrated repeatedly and then processed for the immunocytochemical demonstration of SP using a commercially available monoclonal SP antiserum (Immunonuclear, Stillwater, Okla, USA) and the fluorescence technique described by Coons et al. (1955). For details see Ehinger et al. (1983).

Other whole mounts were kept in a  $\text{Na}_2\text{SO}_4$  solution (24%) at  $37^\circ\text{C}$  overnight. They were then incubated for five hours at room temperature in a solution of acetylthiocholine, iodide (Fluka AG, Buchs, Switzerland) for acetylcholinesterase staining according to the technique of Koelle (1963). The specificity of the staining was confirmed by using Mipafox<sup>R</sup> (N, N-diisopropylphosphorodiamide fluoride,  $4 \times 10^{-6}$  M) (Foa, Stockholm, Sweden) to inhibit butyrylcholinesterase and 284 C 51 (1,5 bis allyldimethyl-ammonium-phenyl) pentan-3-one dibromide,  $10^{-5}$  M (Wellcome Res. Labs., Beckenham, England) to inhibit acetylcholinesterase. The sections were counterstained in eosin and mounted in Permount<sup>R</sup>.

## 2.3 Drugs

Methohexital sodium (Brietal<sup>R</sup>) was obtained from Lilly, Indiana, USA. Capsaicin and TTX were purchased from Sigma, St. Louis, Mo., USA. D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP was generously provided by Dr. J. Hörig, Ferring Arzneimittel, Kiel, FRG. Oxibuprocaine was supplied by Apoteksbolaget, Sweden.

### 3. Results

A few varicose SP fibers could be demonstrated by immunocytochemistry in the cornea (fig. 1a). By contrast, acetylcholinesterase staining revealed a dense network of nerve fibers (fig. 1b). Capsaicin treatment (retrobulbar injection) eliminated neuronal SP from the cornea 48 h later without visibly affecting the cholinesterase-positive nerve fibers (fig. 1c and d). The SP antagonist and the local anaesthetic, oxibuprocaine, had no effect on corneal innervation.

The corneal sensitivity to tactile stimuli, tested with wetted cotton swabs, was completely abolished 2 h after the intravitreal injection of TTX (table 1), and it was abolished immediately after a single topical application of oxibuprocaine. In contrast, the corneal sensitivity to tactile stimuli was completely unaffected by prior retrobulbar injection of capsaicin. The time period studied ranged from 5 h to a week after the injection. Also topical administration of very large amounts of D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP twice daily for 2 months failed to impair the corneal sensitivity to tactile stimuli.

On the whole, the results of the studies of chemogenic pain were similar (table 1). Following administration of tetrodotoxin (intravitreal injection) or oxibuprocaine (topical application) the sensitivity to the chemical stimulus was lost. Pretreatment with capsaicin (retrobulbar injection) had no immediate pain-suppressing effect but transiently suppressed the response (wiping test) to the chemical stimulus 2-3.5 h after pretreatment (fig. 2). However, the rabbits responded to chemogenic pain with blepharospasm at all times; animals pretreated with TTX or oxibuprocaine did not. Pretreatment with D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP was without effect on the chemogenic pain.

The AFR to a standardized trauma (IR of the iris) could be prevented by intravitreal injection of TTX and by topical application of the SP antagonist, as well as by prior retrobulbar injection of capsaicin. Also topically applied oxibuprocaine reduced the AFR when applied repeatedly (every 5 min for 30 min before infrared irradiation) (table 1); one single administration had no effect.

### 4. Discussion

The study shows that the number of SP fibers in the cornea is much lower than the number of acetylcholinesterase-positive fibers. Sectioning the ocular sensory nerve causes the SP fibres to disappear (Keen et al., 1982), indicating that they are part of the sensory system. It is evident that even



if SP is a neurotransmitter associated with nociceptive pathways, it is unlikely to be localized in more than a small fraction of the sensory fibres in the cornea. This agrees well with the observation that only about 20% of the nerve cell bodies in the trigeminal ganglion contain SP (Hökfelt et al., 1975). Moreover, there is evidence that SP is a neurotransmitter in spinal pathways activated by noxious chemical but not by noxious mechanical stimulation (Yaksh et al., 1979; Wright and Roberts, 1980). Capsaicin is thought to excite sensory neurones causing release of SP (Jessell et al., 1978). This is followed by impaired sensory function (Bynke, 1983).

The function of SP in sensory afferents is far from clear. In previous studies on the blink reflex no correlation between SP levels and corneal sensitivity could be established (Keen et al., 1982). Similarly, changes in corneal sensitivity and SP levels do not correlate in capsaicin-treated rats (Lembeck and Donnerer, 1981). In the present study retrobulbar injection of capsaicin transiently reduced the response of the rabbit cornea to chemogenic pain 2-3.5 h later. Four h after capsaicin and up to 5 days later the concentration of SP in the cornea was greatly reduced (Gamse et al., 1981). We could confirm the absence of SP-immunoreactive nerve fibers in the cornea 48 h after capsaicin. Lembeck and Donnerer (1981) and Gamse et al. (1981) gave capsaicin either systemically in large doses or topically several times before testing the sensitivity to chemogenic pain. Lembeck and Donnerer (1981) found that the chemosensitivity of the cornea was diminished 10 min after systemic capsaicin treatment and remained absent for 4 days, the longest time tested. Ten minutes after systemic capsaicin treatment, the SP content of the peripheral nerve was still unchanged, and it was therefore argued that the diminished sensitivity was not caused by depletion of SP (see also Keen et al., 1982). The transient reduction of corneal chemosensitivity after capsaicin pretreatment may reflect a damaging effect of capsaicin on sensory nerve fibers distinct from those producing SP. Capsaicin pretreatment prevents the AFR to local injury, probably as a result of impairment of SP-nerve fiber function. The persisting corneal chemosensitivity (in the absence of SP) after capsaicin pretreatment indicates that SP in the cornea is not necessary for corneal nociception (see also Lembeck and Donnerer, 1981). This may mean either that impulse transmission in the peripheral parts of sensory SP fibers proceeds without the involvement of SP or that the sensory fibers responsible for corneal nociception are distinct from those containing SP. The latter alternative receives indirect support from the fact that



SP fibers represent a minor proportion of the total corneal sensory nerve supply.

Interestingly, the SP antagonist was found to be a potent blocker of neurogenic inflammatory reactions in the eye with no effect on corneal sensitivity.

## 5. Acknowledgements

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Table 1

Effect of various drugs on corneal sensitivity and aqueous flare response (AFR) to local injury in the rabbit

| Drug                        | Number of rabbits | Corneal sensitivity<br>Tactile stimulus <sup>5)</sup> | Chemical stimulus <sup>6)</sup> | AFR to infrared irradiation of the iris |
|-----------------------------|-------------------|-------------------------------------------------------|---------------------------------|-----------------------------------------|
| None                        | 5                 | +                                                     | +                               | +                                       |
| Capsaicin <sup>1)</sup>     | 5                 | +                                                     | +                               | -                                       |
| SP antagonist <sup>2)</sup> | 5                 | +                                                     | +                               | -                                       |
| Tetrodotoxin <sup>3)</sup>  | 3                 | -                                                     | -                               | -                                       |
| Oxibuprocaine <sup>4)</sup> | 3                 | -                                                     | -                               | -                                       |

+, unchanged; -, abolished or greatly reduced.

- 1) The animals were studied 5 h to 1 week after retrobulbar injection of 0.5 ml 1% capsaicin. It should be noted that capsaicin evoked a transient reduction in the responsiveness to chemogenic pain (2-3.5 h after injection).
- 2) D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup> SP (300 nmoles) was applied topically twice a day for 2 months.
- 3) The animals were studied 2 and 4 h after intravitreal injection of 5-10 µg tetrodotoxin.
- 4) Corneal sensitivity was abolished immediately after a single topical application of 50 µl 0.4% oxibuprocaine. Ocular responses to injury was abolished only after repeated applications every 5 min for 30 min.
- 5) Wetted cotton swabs.
- 6) Instillation of a drop of 0.12% capsaicin solution onto the eye.

## Legend to Figure

Fig. 1. Corneal whole mounts processed for demonstration of substance P(SP)-containing nerve fibers (a) and acetylcholinesterase (AChE)-positive nerve fibers (b). The single varicose immunofluorescent SP fiber in (a) contrasts with the dense network of AChE-positive fibers in (b). Two days after treatment with capsaicin (retrobulbar injection) the corneal AChE-positive nerve fibers are unaffected (d), whereas the SP fibers are eliminated (c). 300 X.

Fig. 2. Number of wipings of the eye in response to chemical injury 10 min before and at intervals after retrobulbar injection of capsaicin at zero time,  $P < 0.05$  at 2 h,  $P < 0.01$  at 3.5 h after pretreatment (Student's  $t$ -test).



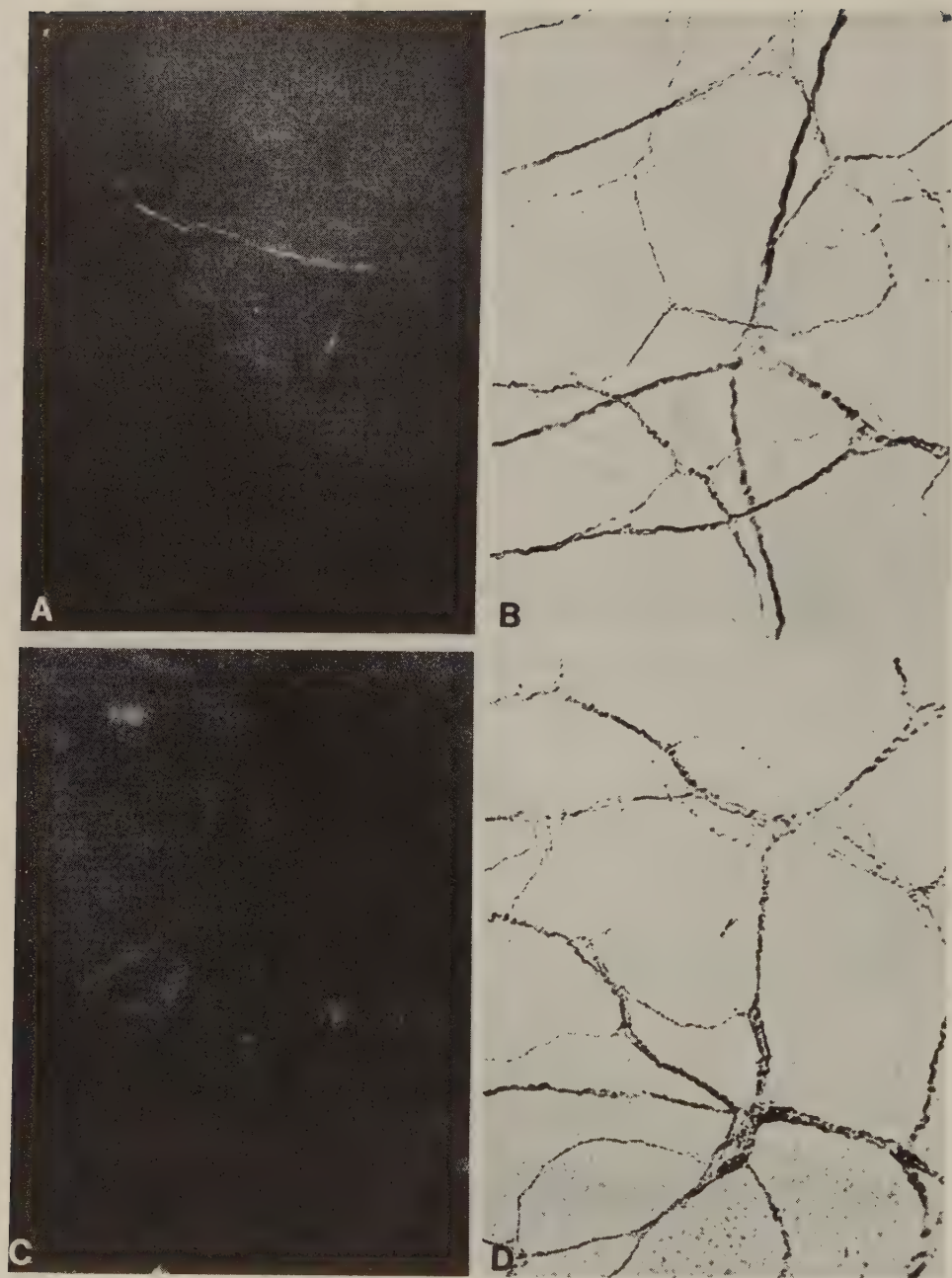


Fig.1

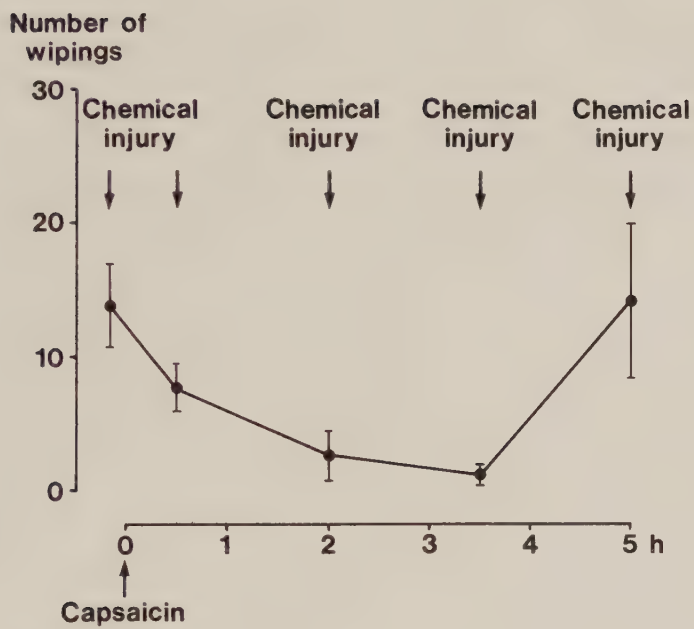


Fig.2



Short title: Substance P antagonists and ocular response to injury.

Substance P antagonists suppress ocular response to injury.

A comparison of five antagonists

G. Bynke, R. Håkanson, J. Hörig and K. Folkers

Department of Experimental Ophthalmology and Pharmacology, University  
of Lund, Lund, Sweden, Ferring Arzneimittel GmbH, Kiel, FRG,  
and Institute of Biomedical Research, University of Texas, Austin,  
Texas, USA

## Summary

Substance P (SP) antagonists are known to suppress the miosis and the disruption of the blood-aqueous barrier evoked by ocular injury in the rabbit. The potency of five different SP antagonists was evaluated by quantitating their effect on the ocular response to a local injury (infrared irradiation of the iris). The pupillary diameter and the disruption of the blood-aqueous barrier were examined. Two undecapeptide and 3 heptapeptide SP analogues were applied topically twice in a dose of 90 nmoles each time, 1.5 and 1 hours before the injury. Dose-response curves were constructed for the two undecapeptides, which were the most potent in suppressing the ocular response; the maximally effective dose was 90 nmoles for both.

## Introduction

There is evidence that all irritant stimuli, except SP, act to release a mediator from sensory nerve fibers in the eye (Butler and Hammond 1980, Håkanson et al. 1983). Since SP is capable of evoking ocular symptoms resembling those seen after local injury (Bill et al 1979, Stjernschantz et al 1981, Holmdahl et al 1981), it has been proposed that the mediator is SP (Zhang et al 1982, Bynke et al 1983a, b).

Recently, SP analogues with SP antagonistic activity have become available (Folkers et al 1981, 1984). In a previous report (Holmdahl et al 1981), one such SP antagonist, (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP, was shown to inhibit the miosis and the disruption of the blood-aqueous barrier following infrared irradiation (IR) of the rabbit iris both when injected intravitreally and applied topically (see also Bynke et al. 1984). Furthermore, topical application of this antagonist reduced the responses to topically applied prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and retrobulbar injection of capsaicin (Bynke et al 1983b).

In the present study, the suppressive effect of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP on the miosis and disruption of the blood-aqueous barrier to IR of the iris was compared with that of 4 novel SP antagonists.

## Materials and methods

Adult pigmented rabbits of mixed strain were used. The blood-aqueous barrier was disrupted by IR of the iris for two minutes (Bengtsson et al. 1975). The course of the barrier damage was followed by photoelectric measurements of the aqueous flare response (AFR) every 30 minutes (Bengtsson et al. 1975). This response is a Tyndall phenomenon in the anterior chamber, reflecting protein leakage across the disrupted blood-aqueous barrier.



A correlation between the AFR and the protein concentration has been established (Anjou and Krakau 1961). The method has the advantage of being atraumatic, permitting recording of responses that cannot be observed by conventional focal illumination. The results are expressed in arbitrary units with reference to a standard (Anjou and Krakau 1961, Bengtsson et al. 1975). The pupillary diameters were measured with a clear plastic ruler under conditions of uniform illumination.

Five SP antagonists were studied (Table 1). All but one were dissolved in sterile 0.9% saline, (Phe<sup>5</sup>, D-Trp<sup>7,9</sup>)SP<sub>5-11</sub> was dissolved in propane-diol/0.9% saline (1:1). In a separate study on a group of 3 rabbits it was shown that this vehicle was without effect in itself. The antagonists were applied topically twice in a volume of 50 µl each time, 1.5 hours and 1 hour before IR of the iris. All rabbits received (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)SP<sub>1-11</sub> onto one eye while the other eye received one of the four other analogues. Following IR of the iris the pupillary diameter and the AFR were recorded. In addition, the effect of various amounts of (D-Arg<sup>1</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>)SP<sub>1-11</sub> (Spantide) alone or of (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)SP<sub>1-11</sub> alone was tested on the left eye of rabbits who received 0.9% saline onto the right eye. After two applications of the antagonist (or saline) both eyes were subjected to IR of the iris and the pupillary diameter and AFR recorded.

### Results

The AFR following IR of the iris was reduced more effectively by (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)SP<sub>1-11</sub> than by any of the other antagonists except for Spantide (Fig. 1). This was so also with respect to pupillary constriction, except that (Phe<sup>5</sup>, D-Trp<sup>7,9</sup>)SP<sub>5-11</sub> produced the same degree of inhibition as (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)SP<sub>1-11</sub> and Spantide. In fact, there was a tendency for

Spantide to reduce both the AFR and the pupillary constriction more effectively than ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )SP<sub>1-11</sub> (Fig. 1). The potency of the latter two antagonists is illustrated by the dose-response curves shown in Fig. 2, which shows only a marginal difference between them.

### Discussion

Previous reports have shown that ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )-SP is effective in reducing the aqueous flare response to a mild physical injury (Holmdahl et al 1981) and to low doses of chemical irritants (Bynke et al 1983a, b). Furthermore, long-term administration of this antagonist completely abolished the AFR to IR of the iris (Bynke et al 1984). The present study compares the potency of five different SP antagonists, previously shown to be effective in vitro (Folkers et al 1984). The miotic response and AFR to IR of the iris were reduced more effectively by ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )-SP<sub>1-11</sub> than by any of the other antagonists except for Spantide. However, the difference between ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )SP<sub>1-11</sub> and Spantide was small. This was surprising in view of the fact that Spantide is about 10 times more potent than ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )SP<sub>1-11</sub> in vitro (Folkers et al 1984). However, the pA<sub>2</sub> values were calculated from studies on the isolated taenia coli of the guinea-pig (Folkers et al. 1984) and may not reflect receptor binding in the eye. Alternatively, the results suggest that the absorption of ( $\underline{\underline{D}}\text{-Pro}^2$ ,  $\underline{\underline{D}}\text{-Trp}^{7,9}$ )SP<sub>1-11</sub> is more efficient or that it has a longer half-life than Spantide.

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Table 1

Amino acid sequences of the SP antagonists tested

|            |                                                                   |
|------------|-------------------------------------------------------------------|
| AP2        | Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub> |
| (Spantide) | D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH <sub>2</sub> |
| AP4        | Arg-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub>                   |
| AP8        | Phe-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH <sub>2</sub>                   |
| AP13       | Arg-Gln-D-Trp-Phe-D-Trp-Leu-Nle-NH <sub>2</sub>                   |

## Legends to Figures

Fig. 1 Miotic and aqueous flare response (AFR) to infrared irradiation (IR) of the iris in rabbits which had been pretreated with two SP antagonists at a time, one in each eye. (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)SP (AP-2) was employed as reference drug and administered to the left eye of all rabbits. The test drug (as indicated in the figure) was applied to the right eye. A standard dose of 90 nmoles of each drug was applied topically twice, 1.5 and 1 hour before IR of the iris. Each value is the mean of 5-8 determinations. Vertical bars give S.E.M. AP-2 was better than AP-4, AP-8 and AP-13 ( $p < 0.001$ ) with respect to AFR; Spantide was slightly better than AP-2 ( $p < 0.05$ ). AP-2 was better than AP-4 ( $p < 0.001$ ) and AP-13 ( $p < 0.01$ ) with respect to miosis; Spantide was better than AP-2 ( $p < 0.01$ ) (two-way analysis of variance).

Fig. 2 Effect of increasing amounts of SP antagonists on the miosis and aqueous flare in response to infrared irradiation of the iris. Each value is the mean of 6 determinations. Vertical bars give S.E.M.



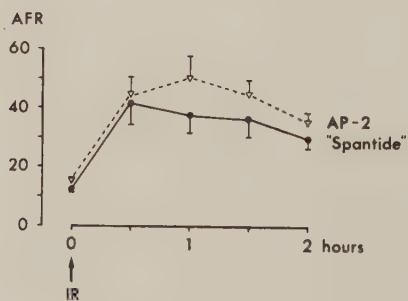
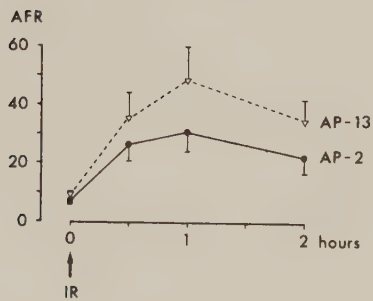
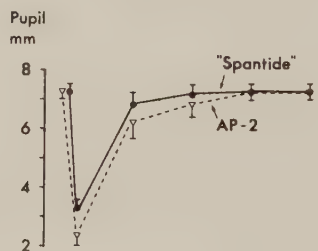
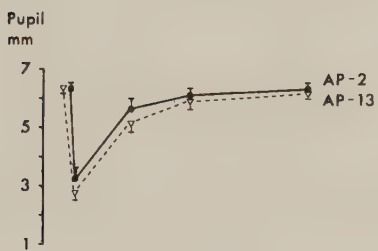
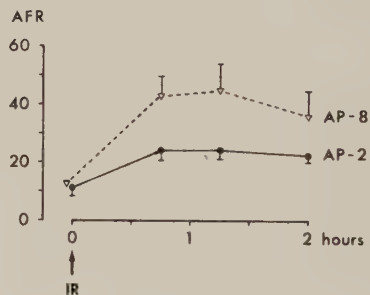
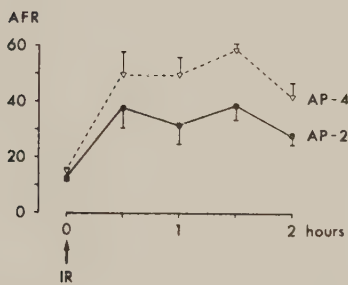
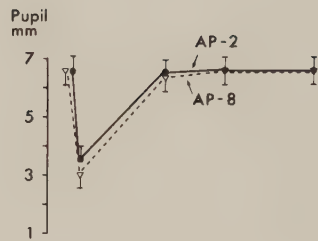
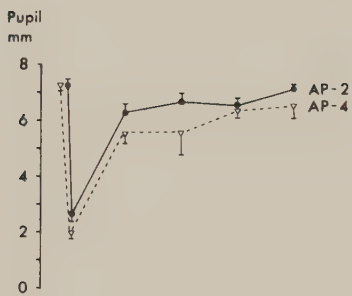
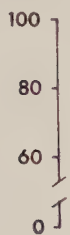
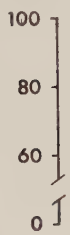


Fig.1

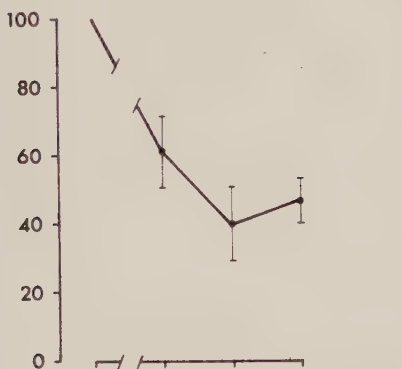
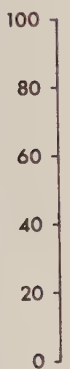
Pupillary  
constriction (%)



Pupillary  
constriction (%)

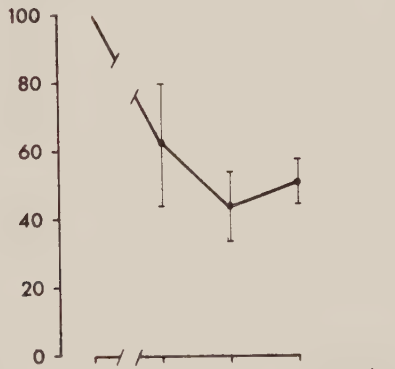
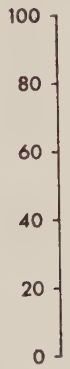


AFR (%)



Spantide

AFR (%)



AP 2

Fig.2



Short title: Hypersensitivity to substance P

Pupillary hypersensitivity to substance P following prolonged treatment  
with tetrodotoxin or substance P antagonists.

G. Bynke, C. Wahlestedt, B. Beding and R. Håkanson  
Departments of Experimental Ophthalmology and Pharmacology,  
University of Lund, Sweden.

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Kiel, FRG, and Ferring Pharmaceuticals, Malmö, Sweden.

## Summary

Pilocarpin contracts the sphincter pupillae muscle via an effect on muscarinic receptors and phenylephrine contracts the dilator pupillae muscle via an effect on  $\alpha$ -adrenergic receptors. These effects are thought to mimic the action of the parasympathetic and sympathetic nervous systems, respectively. Intraocular injection of substance P (SP) produces an atropine-resistant constriction of the pupil. This response is thought to mimic the effect of local sensory reflexes on the sphincter pupillae muscle, engaging SP-containing trigeminal nerve endings. Repeated injections of tetrodotoxin, a general blocker of nervous conduction, over a period of 3 weeks produced hypersensitivity to pilocarpine, phenylephrine and SP in the rabbit eye. These findings support the view that, like acetylcholine and noradrenaline, SP or an SP-like compound acts as a neurotransmitter in the uvea. Also, long-term administration of a substance P antagonist, (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP<sub>1-11</sub> or (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP<sub>5-11</sub>, produced hypersensitivity to SP but not to pilocarpin.

## Introduction

Substance P (SP) is thought to be a neurotransmitter in sensory nerve fibers (Lembeck 1953, Lembeck and Gamse 1982). There is experimental evidence that this is the case also in the eye (Bill et al. 1979, Butler and Hammond 1980, Holmdahl et al. 1981), and that the SP-containing sensory fibers are involved in the mediation of the ocular response to local injury (Holmdahl et al. 1981, Bynke et al. 1983a, b, Bynke et al. 1984).

Injection of substance P (SP) into the anterior or vitreous chamber produces miosis in the rabbit (Bill et al. 1979, Stjernschantz et al. 1981, Holmdahl et al. 1981, Nishiyama et al. 1981). Also in vitro SP evokes a dose-dependent contraction of the isolated sphincter pupillae muscle of the rabbit; the threshold dose of SP is far below that of other known miotic agents (Soloway et al. 1981, Tornqvist et al. 1982). The response to SP is not influenced by blockade of muscarinic, nicotinic, or adrenergic receptors, or by pretreatment with inhibitors of prostaglandin biosynthesis (Cohen et al. 1981, Soloway et al. 1981). Also pretreatment with tetrodotoxin (TTX), a well-known blocker of nervous conduction (Kao 1966), was without effect on the response to SP (Mandahl and Bill 1981, Bynke et al. 1983a). Furthermore, SP retained its miotic effect after trigeminal denervation (Butler and Hammond 1980). These results indicate that SP has a direct effect on the pupillary sphincter.

Recently, a series of SP analogues have been found to block the action of SP in a specific and competitive manner (Folkers et al. 1981, Leander et al. 1981, Håkanson et al. 1982). Two of the SP antagonists, ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP and ( $\text{Arg}^5$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP<sub>5-11</sub> (Folkers et al. 1984) have been shown to inhibit the ocular response to local injury, evoked by a variety of stimuli, including capsaicin (Holmdahl et al. 1981, Bynke et al. 1983a, 1983b, Bynke et al. 1984).



In the present study, attempts were made to produce hypersensitivity to SP by pretreatment with TTX or by long-term treatment with SP antagonists. The inhibiting effect of SP antagonists on the miosis produced by different doses of SP was also tested.

## Materials and methods

### In vivo experiments

Adult pigmented rabbits (1.5-3 kg) of mixed strain were anesthetized with methohexital sodium (Brietal<sup>R</sup>, Lilly, 5 mg/kg) when given intravitreal injections. No anesthesia was administered during the rest of the experiments. The pupillary response to topical application of 0.4, 4 or 40  $\mu$ moles (50  $\mu$ l) pilocarpin hydrochloride (Sigma, St. Louis, Mo, USA) (a muscarinic agonist) or 25, 125 or 250  $\mu$ moles (50  $\mu$ l) phenylephrine (Sigma, St. Louis, Mo, USA) (an  $\alpha$ -adrenergic agonist) or to intravitreal injection of 0.3, 3 or 30 pmoles SP (9  $\mu$ l) (Beckman, Geneva, Switzerland) was measured. In a subsequent series of experiment the pupillary response to 4  $\mu$ moles pilocarpin, 25  $\mu$ moles phenylephrine, or 30 pmoles SP was measured after pretreatment with TTX (Sigma, St. Louis, Mo, USA). TTX was obtained from Sankyo, Tokyo, Japan, and dissolved in redest  $H_2O$ . Five-10  $\mu$ g (10-20  $\mu$ l) TTX was injected into the vitreous chamber of the left eye by means of a Hamilton precision syringe, 3-4 mm posterior to the limbus. The control eye received 10-20  $\mu$ l redest  $H_2O$ . TTX and  $H_2O$  was injected twice a week for 3 weeks, including the day before injection of SP or topical application of pilocarpin or phenylephrine to both eyes.

Topical application of (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP<sub>5-11</sub> or (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP (300 nmoles twice daily) onto the left eye for 2 or 3 months before intravitreal injection of 3, 30 or 300 pmoles (9  $\mu$ l) SP in both eyes or topical application of 4  $\mu$ mole pilocarpin to both eyes. (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP<sub>5-11</sub>

and ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP was a kind gift from Dr. J. Hörig, Ferring Pharmaceuticals, Kiel, FRG.

### In vitro experiments

Adult pigmented rabbits of either sex, weighing 1.5-3 kg, were killed by a blow on the neck and exsanguinated. The eyes were enucleated within 1 min after death, opened by an incision 2-3 mm dorsally to the limbus, followed by excision of the iris from the ciliary margin. The sphincter pupillae muscle (2-3 mm wide) was then opened and mounted vertically on a Perspex holder in a 7 ml organ bath maintained at 33°C. One end was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force displacement transducer for isotonic registration of mechanical activity. The load on the muscle was set at 0.2 g. Before starting the experiments the preparations were allowed to equilibrate for about 90 min. Platinum ring electrodes with a constant electrode distance of 5 mm were placed around the muscle and the electrodes were connected to a Grass S 4C stimulator for field stimulation with square wave pulses (14-17 V over the electrodes, 0.25 ms duration). The muscles were stimulated with trains of pulses lasting 10 s, the pulse frequency being 20 Hz. The resting period between stimulations was at least 5 min. Three reproducible contractions of each preparation were used for calculations of the ratio between the amplitude of the slow and the fast contraction. The ratios are expressed as means  $\pm$  S.E.M. The mechanical activity of the preparation was continuously recorded on a Grass model 7 or model 5 polygraph. The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 133,  $\text{NaHCO}_3$  16.3, KCl 4.7,  $\text{MgCl}_2$  1.0,  $\text{NaH}_2\text{PO}_4$  1.4,  $\text{CaCl}_2$  2.5 and glucose 7.8. The solution was bubbled with a gas mixture of 7%  $\text{CO}_2$  in  $\text{O}_2$  giving a pH of 7.2-7.3.

Both iris sphincter muscles from 5 rabbits pretreated with (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP topically onto the left eye (300 nmoles twice daily for 2 months) were tested in parallel with iris sphincter muscles from 5 age-matched control rabbits. The experiments were carried out 2-4 days after the last application of (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP.

## Results

### Effect on the pupil

The effects of SP, pilocarpin and phenylephrine on the pupil are illustrated in Fig. 1. The pupillary constriction evoked by SP and pilocarpin was enhanced after pretreatment with TTX (Fig. 2). Also the pupillary dilatation to phenylephrine was more pronounced in the TTX-treated eye than in the control eye (Fig. 2).

During long-term treatment with (D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>)-SP or (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP<sub>5-11</sub> the pupillary response to intravitreal SP was abolished or reduced (Fig. 3). A few days after termination of treatment with SP antagonist the response to SP but not to pilocarpin was enhanced (Fig. 4).

### Studies on the isolated iris sphincter muscle

The response of the sphincter muscle of a control rabbit to electrical stimulation was an initial twitch with a high amplitude, followed by a slow contraction with a lower amplitude (Fig. 5). The twitch could be blocked by atropine, the slow contraction by the SP antagonists (not shown). Following treatment with the SP antagonist for 2 months and termination of treatment for 2-4 days the slow contraction became much more prominent both in the treated sphincter and in the contralateral sphincter. The amplitude of the slow contraction was compared to the amplitude of the twitch in muscles from control rabbits and from rabbits pretreated with the SP antagonist (Fig. 5). The ratio of the amplitudes was  $0.78 \pm 0.03$  (S.E.M.) ( $n = 15$ ) in control

sphincters as compared with  $1.09 \pm 0.04$  ( $n = 15$ ) ( $p < 0.001$ ) in sphincters treated with the SP antagonist and  $1.41 \pm 0.06$  ( $p < 0.001$ ) in the contralateral sphincter.

### Discussion

SP is thought to be a mediator in sensory nerve endings (Lembeck and Gamse 1982) and held to be responsible for the neurogenic component in the response to ocular injury (Holmdahl et al. 1981). TTX is a blocker of nervous impulse conduction (Kao 1966) and treatment with TTX over a period of time might be expected to cause denervation hypersensitivity. In fact, prolonged exposure to TTX enhanced the pupillary responses to pilocarpine, neosynephrine and SP. The results support the view that, like acetylcholine and noradrenaline, SP is a neurotransmitter in the iris. Long-term treatment with an SP antagonist enhanced the pupillary response to SP but not to pilocarpin.

Electrical stimulation of the isolated iris sphincter muscle evoked a rapid twitch, followed by a slow contraction. In confirmation of previous reports the twitch could be blocked by atropine (Ueda et al. 1981), and the slow contraction could be blocked by SP antagonists (Leander et al. 1981, Björkroth, 1983). The results of the present study revealed a relative enhancement of the slow contraction following long-term pretreatment with the SP antagonist. This is compatible with the development of hypersensitivity following long-term receptor blockade. It could be argued that the relative enhancement reflects instead a reduced twitch. However, this is unlikely since the SP antagonist does not interfere with cholinergic mechanisms (Leander et al. 1981). Interestingly, the enhancement of the slow contraction was observed not only in the sphincter muscle from the treated eye but also from the contralateral eye, suggesting either a direct passage of the SP antagonist from one eye to the other or a centrally mediated adjustment. We have no explanation for the fact that the enhancement of the slow contraction was more pro-

nounced in the sphincter muscle from the contralateral eye than from the treated eye. It cannot be excluded that SP antagonist remained in the treated eye, but not in the contralateral eye, at the time of the experiments (2-4 days after termination of treatment) (see also Bynke et al. 1984). Similarly, after long-term treatment with SP antagonists, the miosis produced by a low dose of SP was greatly reduced in both the treated and untreated eye compared with a control group. This suggests that with time the SP antagonists passes over to the contralateral eye in amounts sufficient to cause effects. The miotic response to a large dose of SP was inhibited only in the treated eye. The results are compatible with the view that SP, like acetylcholine and noradrenaline, acts as a neurotransmitter in the rabbit eye.

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## Legends to figures

Fig. 1. a) Pupillary constriction observed 3.5 h after injection of SP ( $n = 9$  for each dose). b) Pupillary constriction observed 0.5 h after application of pilocarpin ( $n = 10$  for each dose). c) Pupillary dilatation observed 5 min after application of phenylephrine ( $n = 8-10$  for each dose). The values are given in mm (means  $\pm$  S.E.M.).

Fig. 2. Maximal pupillary constriction (mm) (mean  $\pm$  S.E.M.) in response to 30 pmoles SP or to 4  $\mu$ moles pilocarpin after long-term TTX pretreatment of the left eye. The pupillary constriction of the TTX-treated eye to SP or pilocarpin was significantly enhanced,  $p < 0.01$  and  $p < 0.001$ , respectively (Student's t-test). Maximal pupillary dilatation (mm) (mean  $\pm$  S.E.M.) in response to phenylephrine to both eyes after long-term TTX pretreatment of the left eye. The pupillary dilatation of the TTX-treated eye was significantly enhanced,  $p < 0.0005$  (Student's t-test).

Fig. 3. (left) Maximal pupillary constriction in response to 300 pmoles SP 3 months after daily topical application of ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP to the left eye,  $p < 0.025$ , (Students t-test). SP was administered 3 h after the last application of the SP antagonist. (right) Maximal pupillary constriction to 3 pmoles SP 2-3 months after daily topical application of ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP or ( $\text{Arg}^5$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP to the left eye. SP was administered 2-3 h after the last application of the antagonist. Compared with the response produced by the the same dose in control rabbits, the pupillary constriction of both the left and the right eye was significantly lower,  $p < 0.005$  and  $p \leq 0.005$ , respectively (Student's t-test) (see Fig. 1). The values are given in mm (means  $\pm$  S.E.M.).

Fig. 4. Maximal pupillary constriction (mm) (Mean  $\pm$  S.E.M.) to 30 pmoles SP or to 4  $\mu$ moles pilocarpin 2 months after daily topical application of (Arg<sup>5</sup>, D-Trp<sup>7,9</sup>)-SP to the left eye. The miotics were given 2 days after termination of treatment with the SP antagonist. The pupillary constriction of the left eye to SP was significantly enhanced,  $p < 0.005$  (Student's t-test). There was no difference in the pilocarpin-induced pupillary constriction between the left and the right eye.

Fig. 5. Representative tracings of the responses to electrical field stimulation of isolated iris sphincter muscle from rabbit showing a rapid twitch, followed by a slow contraction. A. Iris sphincter from an eye treated with SP antagonist for 2 months, followed by 2-4 days without treatment. B. Iris sphincter from the contralateral eye. C. Iris sphincter from a control rabbit not exposed to SP antagonist.

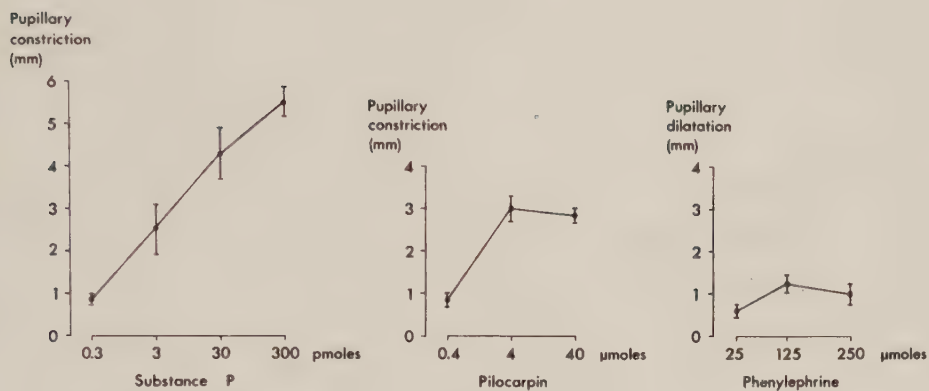


Fig.1

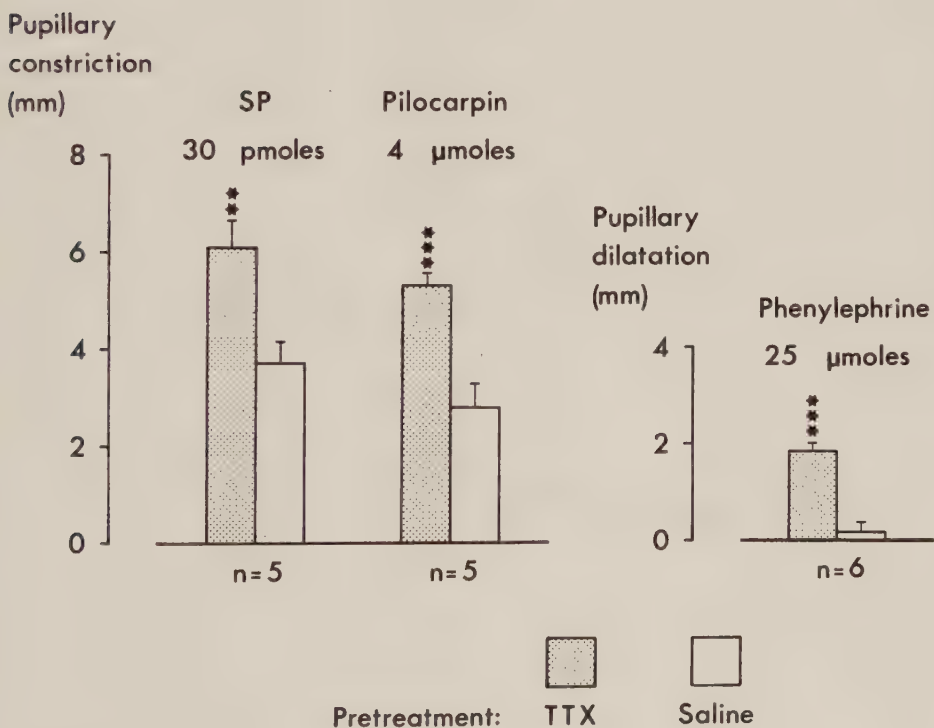


Fig.2

Pupillary  
constriction  
(mm)

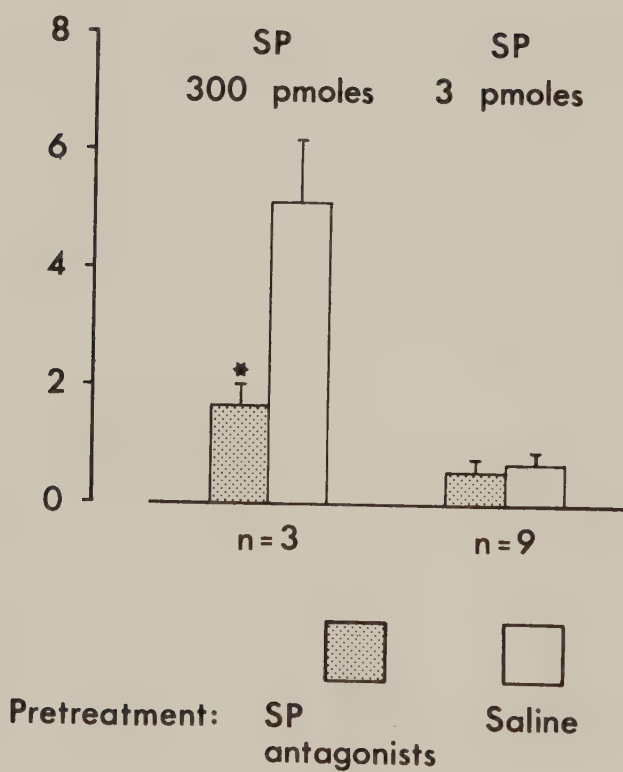


Fig.3

Pupillary  
constriction  
(mm)

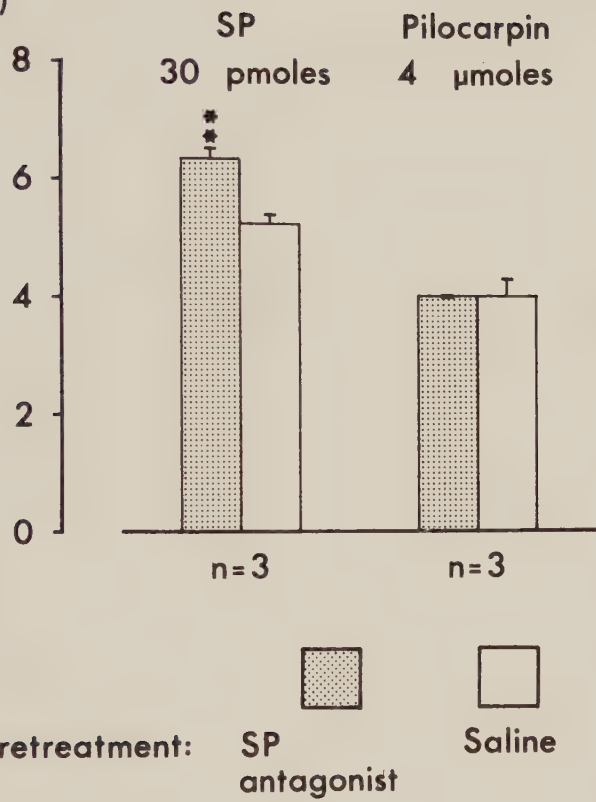
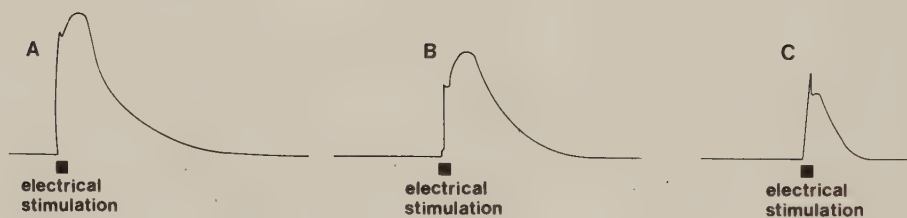


Fig.4





**Fig.5**

Sympathectomy enhances the substance P-mediated response to  
infrared irradiation of the rabbit iris

by

G. Bynke, A. Bruun and R. Håkanson

Departments of Experimental Ophthalmology and Pharmacology,  
University of Lund, Sweden

Short title: Sympathectomy and ocular responses to injury

## Summary

Rabbits were subjected to infrared irradiation of the iris one month after unilateral cervical sympathectomy. The resulting breakdown of the blood-aqueous barrier, reflected in aqueous flare and measured photo-electrically, was greatly enhanced on the sympathectomized side. In contrast, the response to intravitreally injected substance P (SP) was the same in both eyes. The enhancement of the response to infrared irradiation could be abolished by pretreatment with an SP antagonist, ( $\underline{\underline{D}}$ -Pro<sup>2</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>)-SP.

## Introduction

The response of the rabbit eye to laser irradiation of the pigmented iris appears to be mediated partly by E-type prostaglandins (PGs) and partly by a non-cholinergic neurogenic factor (Unger et al 1974, 1977). Similar mechanisms seem to be involved in the break-down of the blood-aqueous barrier evoked by infrared irradiation (IR) of the rabbit iris, since it is reduced after topical or retrobulbar anaesthesia (Dyster-Aas 1965) and after indomethacin (Bengtsson et al 1975). However, Butler and Hammond (1980) emphasized the key role of the neurogenic pathway in the response to all types of noxious stimuli, since trigeminal denervation was found to abolish the response not only to laser irradiation of the iris (Butler et al 1980b) but also to  $\text{PGE}_1$ . The response to substance P (SP) was not abolished. SP has been put forward as a candidate mediator of the ocular response to injury, since it evokes symptoms of ocular injury (Bill et al 1979, Stjernschantz et al 1981, Holmdahl et al 1981) and since it exists in the trigeminal nerve from which it is released into the aqueous humour upon stimulation (Bill et al 1979). Moreover, destruction of the trigeminal ganglion is followed by reduced SP levels in the uvea (Butler et al 1980a). Also, a substance P antagonist, ( $\underline{\text{D}}\text{-Pro}^2$ ,  $\underline{\text{D}}\text{-Trp}^{7,9}$ )-SP, has been shown to reduce the breakdown of the blood-aqueous barrier to IR of the iris (Holmdahl et al 1981) and to  $\text{PGE}_2$  (Bynke et al 1983) and  $\text{PGE}_1$  (Mandahl and Bill 1983).

Following sympathectomy, the ocular response to laser irradiation of the iris is increased (Unger 1977, Unger et al. 1981). Concomitantly, the SP levels in the uvea of the rabbit are raised significantly (Cole et al 1983). This gives indirect support to the theory that SP plays a key role in the ocular response to injury.

In the present study, the breakdown of the blood-aqueous barrier caused by IR of the iris was studied after sympathectomy and the involvement of SP was tested by the use of an SP antagonist.

#### Materials and methods

Five adult pigmented rabbits (3-5 kg) underwent extirpation of the right cervical ganglion during pentobarbital (Mebumal<sup>R</sup>) anaesthesia. Successful sympathectomy was indicated 3 weeks later by the supersensitivity of the mydriatic response to topically applied phenylephrine (Neosynephrine<sup>R</sup>). One month after sympathectomy the rabbits were subjected to IR of the iris of both eyes for 2 minutes. The time course of the consequent barrier damage was followed by photo-electric measurement (Bengtsson et al 1975) of the aqueous flare response (AFR) every 30 minutes. This response is a Tyndall phenomenon, reflecting protein leakage into the anterior chamber. A correlation between the AFR and the protein concentration has been established (Anjou and Krakau 1961). The results are expressed in arbitrary units with reference to a standard. A week later the right eye was pretreated with 300 nmoles ( $\underline{\underline{D}}$ -Pro<sup>2</sup>,  $\underline{\underline{D}}$ -Trp<sup>7,9</sup>)-SP, topically every 15 min for 1.5 hours before IR of the iris of both eyes, and the AFR was measured. One month later the same animals were given 30 pmole (9  $\mu$ l) SP intravitreally to both eyes.

#### Results

The pupil of the sympathectomized eye was significantly smaller than that in the normal eye before IR of the iris,  $4.9 \text{ mm} \pm 0.33$  versus  $6.5 \pm 0.22$ ,  $p < 0.0025$  (Students t-test). There was no significant difference in the IR-induced pupillary constriction between the right and the left eye (Students t-test) (Fig. 1A). The AFR following IR of the iris was significant-

ly greater in the right eye (Fig. 1A). The miotic response to intravitreally injected SP was the same in both eyes, the pupillary diameter after 4.5 h being  $3.5 \text{ mm} \pm 0.79 \text{ mm}$  in the right eye and  $2.7 \text{ mm} \pm 0.62 \text{ mm}$  in the left eye. After pretreatment with ( $\underline{\text{D}}\text{-Pro}^2$ ,  $\underline{\text{D}}\text{-Trp}^{7,9}$ )-SP of the right eye there was no longer any difference between the AFR following IR of the iris (Fig. 1B). The miotic response to IR was not significantly affected by pretreatment with the SP antagonist.

## Discussion

Sympathectomy is known to increase the SP levels in the uvea (Cole et al. 1983) and to enhance the response to ocular injury (Unger 1977, Unger et al. 1981). This is suggestive of a causal relationship, SP being a proposed mediator of the inflammatory response. As anticipated, the AFR evoked by IR of the iris was enhanced one month after sympathectomy. The enhancement could be abolished by pretreatment with ( $\underline{\text{D}}\text{-Pro}^2$ ,  $\underline{\text{D}}\text{-Trp}^{7,9}$ )-SP. The effect of intravitreally applied SP was the same in the sympathectomized and in the control eye. Hence, the enhancement following sympathectomy does not reflect an increased sensitivity to SP but rather support the view that SP is the mediator of the ocular response to injury and that the SP nerve supply to the uvea is increased following sympathectomy.

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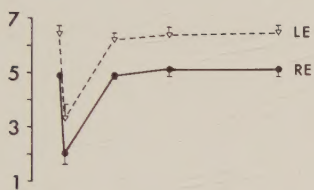
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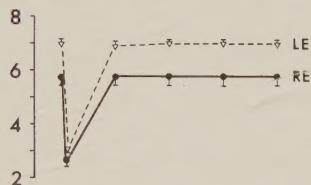
## Legend to figure

Fig. 1A. Aqueous flare response (AFR) (mean  $\pm$  SEM) to infrared irradiation (IR) of the iris of the left eye (LE) and the right eye (RE) in rabbits one month after cervical sympathectomy of the right side. The difference between the eyes is highly significant, ( $p < 0.001$ , analysis of variance, two-way). B. One week later, the right eye was pretreated with repeated applications of ( $\underline{\text{D-Pro}}^2$ ,  $\underline{\text{D-Trp}}^{7,9}$ )-SP before IR of the iris of both eyes. There was no longer any difference between the eyes ( $p > 0.05$ , analysis of variance, two-way).

Pupillary  
diameter (mm)



Pupillary  
diameter (mm)



AFR

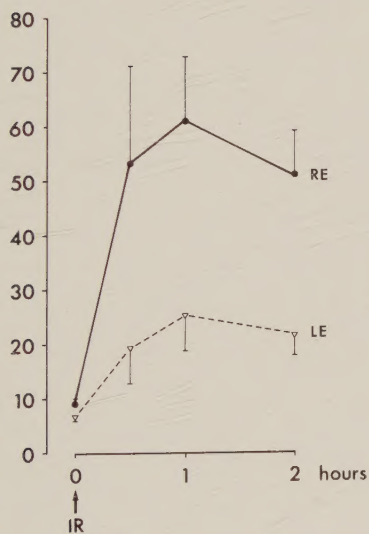


Fig.1 A

AFR

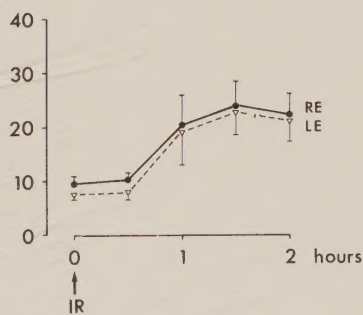


Fig.1 B







